610.5
A595
M497

U. of MICH. MEDICAL BULLETIN

MAR 7 - 1951

ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

REDACTORES:

E. MUSTAKALLIO (TURKU)
U. UOTILA (HELSINKI)
ARMAS VARTIAINEN (HELSINKI)
ALVAR WILSKA (HELSINKI)
A. I. VIRTANEN (HELSINKI)

EDITOR

K. O. RENKONEN

REDIGENDA CURAVIT

M. TUOMIOJA

VOL. 28 1950 FASC. 2

MERCATORIN KIRJAPAINO
HELSINKI, FINLAND

UNIV. OF MICH.

Annales Medicinae Experimentalis et Biologiae Fenniae

is a direct continuation of the *Acta Societatis Medicorum Fennicae »Duodecim»*, 1919—1930 (Vols. I—XII) and the *Acta Societatis Medicorum Fennicae »Duodecim», Ser. A*, 1931—1946 (Vols. XIII—XXIV).

The journal is published by the Finnish Medical Society »Duodecim» with the object of providing an opportunity to publish articles on experimental medicine and on related biological subjects.

Each number of the journal contains 80—100 pages, three numbers forming one volume. Articles are accepted for publication with the understanding that they are original contributions never previously published. The manuscripts should be in English, French or German, and typewritten. They should not exceed a total length of 24 pages, and there should be a short summary at the end of the article.

The subscription price is Fmks 400 per volume in Finland and $ 4 or Swedish Crowns 15.00 in foreign countries. More extensive works, published as supplements, the subscribers receive free of charge.

Address for subscription, exchange of reviews, and all communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finland.

Annales Medicinae Experimentalis et Biologiae Fenniae

est une suite directe des revues *Acta Societatis Medicorum Fennicae »Duodecim»* 1919—1930 (V. I—XII) et *Acta Societatis Medicorum Fennicae »Duodecim»* Ser. A, 1931—1946 (V. XIII—XXIV).

La revue est éditée par la Société de médecins finnois »Duodecim», et a pour but d'offrir l'occasion de publier des recherches scientifiques appartenant à la médicine expérimentale et à la biologie étroitement liée avec la médicine.

La revue paraît en cahiers comprenant à peu près 80—100 pages. 3 cahiers forment un volume. Les articles destinés à la revue ne doivent pas être publiés ailleurs. Les manuscrits dactylographiés doivent être rédigés en français, allemand ou anglais et leur longeur totale ne doit pas en général dépasser 24 pages. Un court résumé doit se trouver à la fin.

Le prix de l'abonnement pour la Finlande est marcs 400, pour l'étranger $ 4 ou couronnes suédoises 15 par volume. Les travaux plus étendus, qui seront éventuellement publiés en suppléments, seront distribués gratuitement aux abonnés.

Adresse pour abonnement, échange de journaux et toutes autres communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finlande.

Annales Medicinae Experimentalis et Biologiae Fenniae

sind eine direkte Fortsetzung der *Acta Societatis Medicorum Fennicae »Duodecim»*, 1919—1930 (Vol. I—XII) und der *Acta Societatis Medicorum Fennicae »Duodecim», Ser. A*, 1931—1946 (Vol. XIII—XXIV).

Die Zeitschrift wird vom *Finnischem Ärzteverein »Duodecim»* herausgegeben und hat zur Aufgabe wissenschaftliche Untersuchungen aus dem Gebiete der experimentellen Medizin und sich daran schliessenden biologischen Forschungsgebieten aufzunehmen.

Jede Nummer der Zeitschrift erscheint in einem Umfange von 80—100 Druckseiten. Drei Nummern bilden ein Volumen. In die Zeitschrift werden nur Originalarbeiten aufgenommen, die nicht früher veröffentlicht worden sind. Die mit Schreibmaschiene geschriebenen Manuskripte sind in deutscher, englischer oder französischer Sprache einzusenden und sollen im allgemeinen nicht mehr als 24 Druckseiten betragen. Jede Untersuchung ist durch eine kurze Zusammenfassung abzuschliessen.

Der Bezugspreis beträgt für das Innland 400 Fmk und fürs Ausland 4 $ bzw. 15 Schwedenkr. Umfangreichere Untersuchungen, die als Supplemente herausgegeben werden können, erhalten die Abonnenten abgabefrei.

Bezugsadresse sowie Anschrift für Austausch von Zeitschriften und alle anderen Mitteilungen:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finnland.

Medical
Library
Duval
3-12-51

610.5
A595
M497

MEAN ERROR IN ANTISTREPTOLYSIN DETERMINATIONS

By

NILS OKER-BLOM

(Received for publication August 15, 1949)

At the Department of Serology and Bacteriology, University of Helsinki, the mean error in a single antistreptolysin determination has, according to Kalbak (1), previously been estimated to ± 20 per cent. Nevertheless, control titrations have shown that the AST value by repeated determinations may deviate from the original by as much as 100 per cent. In these extreme cases the cause may possibly be found in the serum itself, but in most cases the deviation certainly depends on the methods used.

Lagercrantz (2) has recently discussed this problem. He says that the errors of titration by laboratory assistants differ greatly. Since other factors, in spite of nearly uniform technique, may also influence results in different laboratories, it seems advisable to determine the mean error in AST titrations separately for each laboratory and for each laboratory assistant.

In the present study, the mean error in our AST determinations has been calculated in two different ways. First, twenty sera were titrated ten times each. In this case the laboratory assistant knew the object of the investigation, and one therefore could expect the greatest possible precision. Second, fifty sera were titrated three times each without the laboratory assistant knowing the object of the investigation.

MATERIAL AND METHODS

The sera were heated to 56° C for half an hour, and unless immediately used they were stored at −8° to −15° C.

The AST determinations were performed with streptolysin

freshly reduced for each time and according to a previously described method (3).

RESULTS

When calculated in the former way the mean error was ±29 per cent. In this case the error for each serum was calculated from the ten determinations according to the formula:

$$\sigma p = \sqrt{\frac{\Sigma (x p - mp)^2}{k-1}}$$

σp = the mean error for each serum
xp = AST values for the serum p
mp = mean for the ten AST values
k = number of determinations for each serum.

The final mean error was then calculated from the errors for each serum according to the formula:

$$\sigma (x)\ {}^0/_0 = \frac{1}{n} \Sigma \sigma p\ {}^0/_0$$

In the latter case the mean error was calculated from the logarithms of the AST values according to the formula:

$$\sigma (\log x) = \sqrt{\frac{\Sigma (\log x p - m p)^2}{n\ (k-1)}}$$

$$\text{and } \sigma (x)\ {}^0/_0 \sim 2{,}3 \times \sigma (\log x)$$

where

xp = AST values for the serum p

$mp = \dfrac{\Sigma \log x p}{k}$ = mean for the log. of the three AST values for each serum

k = number of determinations for each serum

n = number ot sera

In this case the mean error was ± 27 ± 2 per cent.

CONCLUSION

According to this investigation the mean error for AST determinations seems to be, on the whole, the same whether the labora-

tory assistant knows the purpose of the investigation or whether he does not, about ± 30 per cent.

These results are to some extent surprising as, based on earlier investigations, one would expect the mean error to be smaller if the laboratory assistant is aware of the purpose of the investigation. It might, in this case, possibly be due to the fact that this particular assistant is incapable of exceeding this mean error.

In any case a mean error of ± 30 per cent should be considered correct when it is a question of routine investigations in this laboratory, and should also be considered when calculating changes in the AST titer.

REFERENCES

1. Kalbak, K.: Dissert. København 1942.
2. Lagercrantz, R.: Nord. Med. 1949:*40:*2143.
3. Oker-Blom, N.: Finlands Läkartidn. 1948: *19:* 546.

FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI, AND
THE INSTITUTE OF PHYSICAL CHEMISTRY, ÅBO AKADEMI

SOME REMARKS ON THE »SOLVENT EFFECT» AND THE APPARENT »ANTICARCINOGENIC ACTION» OF LANOLIN

By

KAI SETÄLÄ and PER EKWALL

(Received for publication November 13, 1949)

It has been suggested in the literature that addition of anhydrous lanolin (*i.e.*, anhydrous wool fat, which presumably represents chiefly the secretion of the sebaceous glands of the sheep) may lead to a reduction in neoplastic response of the tissues (19, 20, 21, 23, 24, 25). It was found that the lanolin solution of 20-methylcholanthrene generally failed to produce the carcinogenic effect of this compound when applied to the skin of mice (19, 20, 21). The carcinogen dissolved in lanolin did not even induce any of the other preliminary changes that follow the application of the acetone or benzene solution (*i.e.*, epilation of the skin, destruction of the sebaceous elements, or a non-neoplastic epithelial hyperplasia) (20). Examination in ultraviolet light, however, has revealed that the skin of mouse absorbs 20-methylcholanthrene from its solution in melted lanolin (40—45° C. at the time of use). This appears in the sebaceous elements and in the keratin layer. After a single painting this fluorescence disappears in 2 to 3 days, which is much quicker than when a benzene solution is applied (20). Other fatty substances have also been studied as solvents (23, 24, 25), but lanolin had the most pronounced effect in diminishing carcinogenic potency. In view of this apparently specific inhibition of carcinogenesis, the use of lanolin (as »anticarcinogenic» agent) has been recommended as a preventive against occupational

cancer (25). Simpson and Cramer (20, 21), and Simpson, Carruthers and Cramer (19) suggest that the inhibitory effect of lanolin is not a result of (*1*) the inactivation of the carcinogen by the lanolin, (*2*) the prevention of the carcinogen from reaching the epithelial cells(?), or (*3*) failure of the carcinogen to persist long enough *in situ*. The authors mentioned (21) put forth the tentative hypothesis that »unaltered methylcholanthrene is a sensitizing agent, itself non-carcinogenic, that prepares the skin for subsequent action by metabolite derivatives of the carcinogen, or by substances formed in the tissues as a result of exposure to these metabolic products», with the implication that lanolin specifically interferes with this mechanism.

Furthermore, Simpson and Cramer (21) stated that the manifestation of the carcinogenic effect, including the earliest changes initiating that effect, depends on the nature of the solvent. For convenience of description the group of solvents such as benzene, chloroform, and acetone, in which 20-methylcholanthrene is carcinogenically active, will be described as forming »active» solutions; those in which this carcinogen is carcinogenically inactive will be referred to as giving »inactive» solutions. The first effect of a carcinogen when applied in an active solution is to destroy the sebaceous elements. The glands remain intact when the carcinogen is applied in an »inactive» solution (20).

Berenblum and Schoental (1) confirmed the opinion that a striking diminution is observed in the carcinogenic potency of 20-methylcholanthrene as well as 9.10-dimethyl-1.2-benzanthracene when lanolin was used as carrier and spreader for these compounds. They reported that when a benzene solution of 20-methylcholanthrene is applied to the skin the benzene evaporates immediately, leaving behind the carcinogen dissolved in the minute amounts of sebum normally present on the skin. The *effective* concentration of the carcinogen will therefore be much higher than the *original* concentration in the benzene. But when using a non-volatile solvent, such as lanolin, the effective concentration practically corresponds to that of the solution as originally applied. Berenblum and Schoental showed that when liquid paraffin, which like lanolin is non-volatile, is employed as solvent for the carcinogen, a similar diminution in the carcinogenic potency is observed when the concentration of the carcinogen is low, but this no longer applies

when the concentration is increased. The whole phenomenon thus reduces to a concentration effect. In their conclusion Berenblum and Schoental stated that in the case of a solution in lanolin a concentration of 0.3 per cent 20-methylcholanthrene probably is a subthreshold for effective carcinogenesis.

The importance of the »adequate concentration» of the carcinogen in skin carcinogenesis has been reported in some re-examinations also. Among others, Berg (2) has shown that in order to obtain the same fluorescence intensity in the epithelial cells with lanolin solution of benzpyrene as with a 0.025 per cent solution of benzpyrene in acetone, the concentration of benzpyrene must be 0.5 per cent. The carcinogenic action of the hydrocarbon parallels the effective concentration in the epithelial cells. Berg assumes that this strengthens the opinion that the special diffusion and solubility relations existing in the skin when fatty materials are used as solvents is the reason for the apparent decrease in the carcinogenic effect when lanolin is used as solvent and in the first place this is a question of a concentration effect.

It is evident that the concentration of the carcinogen plays an important role in chemical carcinogenesis. However, it is likewise evident that there are several other factors also contributing to the carcinogenic response of tissue. *Very important in this connection is the physical and chemical character of the solvent* (*i.e., the »spreader«*) *for the carcinogen.* It is to be noted that the nature of this »solvent effect» still remains almost unexplained. For the elucidation of this question it would in the first place be of value if carcinogenic hydrocarbons could be applied in various solvents, both volatile and non-volatile, possessing different and well-defined physical and chemical characteristics. A biological truth seems to be that *the chemical reactions associated with life take place* — at least to some extent — *between substances in aqueous solution and aqueous colloidal systems, or at interfaces between them.* It would therefore be of special significance to find carriers that are both water- and fat-soluble or are able to dissolve both water- and fat-soluble substances (16). Using well-defined and well-known carriers of this type, it should in our opinion be possible to obtain further information of the importance of the solvent in chemical carcinogenesis and of

how the carcinogenic hydrocarbons actually act. For several years (since 1945; 16) we have carried out experiments along different lines when studying the significance of certain factors in chemical carcinogenesis, and several of the observations have already been reported.

»Carbowax 1500» was the first compound that was found to possess expected specific characteristics. It was found — as a result of fluorescence studies — that carcinogenic hydrocarbons (20-methylcholanthrene, 1.2.5.6-dibenzanthracene, 9.10-dimethyl-1.2-benzanthracene) dissolved in »Carbowax» were able to permeate into the tissues easily (12, 14). Further, it was found that the treatment of mice with these carcinogenic hydrocarbons (*e.g.*, 20-methylcholanthrene, 9.10-dimethyl-1.2-benzanthracene) resulted in multiple progressively growing tumours (13, 17). The »Carbowax»-group (trade mark) consists of polyethylene glycols of high molecular weight. These compounds have a particular characteristic: *they are readily soluble in water.* Polyethylene glycols also have *the property of lowering surface tension*, and are at the same time *wetting agents.* Thus, the »Carbowax»group represents a new type of solvent for carcinogenic hydrocarbons differing in principle from both the fat-solvent-type (*e.g.*, benzene, acetone, dioxane) and from the »lanolin»-type.

Aqueous solutions of association colloids furnished another type of carriers for carcinogenic hydrocarbons (4, 5, 6, 7, 18). To the group of association colloids belong various *detergents* and *wetting agents*, and also several substances found in the living organism. The simple molecules and ions of these substances associate in aqueous solution to give micelles, and these micelles are able to bring certain quantities of carcinogenic hydrocarbons into solution. The resulting aqueous solutions are both clear and stable, and furthermore usually have low surface tensions. They therefore just as well spread over the skin and penetrate it to some extent (5). Investigations with the fluorescence microscope revealed that the solubilized carcinogen thus also enters the tissues. Treatment of the mouse skin with these solutions resulted in multiple, progressively growing warts (6, 7, 18). The aqueous association colloid solutions also differ in principle both physically and chemically from the »ordinary» fat solvents and also from the fats and oils used as solvents in chemical carcinogenesis.

Because lanolin is not a pure chemical compound, but a mixture

of esters and free alcoholic compounds we have not considered it a suitable carrier for the carcinogens in our systematic studies of the »solvent effect». When we studied new carriers with definite chemical and physical properties in our experiments, certain phenomena were encountered, which were to some extent similar to those observed when lanolin was used. We have therefore conducted a number of experiments using lanolin as solvent for the carcinogens. Some of these observations are briefly dealt with in the present report along with certain other observations. We do not, however, want to draw any final conclusions as to whether lanolin *per se* has any »anticarcinogenic action» or not.

EXPERIMENTAL PROCEDURES

The animals used were white mice of the same anonymous strain, of both sexes used during several years in our experiments. The total number of animals, in the present study, was about five hundred.

The mice were painted three times a week on a large area of the back. Prior to each application the hair was clipped with scissors. Careful note was made of the general condition of the animals, the survivor rate in each group, the epilation of the skin, and the development of tumours.

As »carriers» the following compounds were used: (*1*) anhydrous lanolin; (*2*) aqueous lanolin; (*3*) acetone, and (*4*) dioxane; (*5*) *unmelted* »Carbowax 1500»; (*6*) aqueous solution of »Triton NE»; (*7*) aqueous solution of sodium cholate; (*8*) aqueous solution of sodium myristyl sulphate, and (*9*) aqueous solution of sodium oleate.

The carcinogen used was 9.10-dimethyl-1.2-benzanthracene (Eastman Kodak Co., Rochester, N.Y.). The concentration of the carcinogen varied from 0.25 to 0.3 per cent in lanolin and »Carbowax» and from 0.03 to 0.3 per cent in acetone and dioxane. The »Triton» solution contained about 0.07 per cent carcinogen, and the aqueous solutions of »Duponol», sodium cholate and sodium oleate about 0.03 per cent respectively.

The fluorescence experiments were carried out in the same manner as in our previous works (12, 14). It may be mentioned that the carcinogen 9.10-dimethyl-1.2-benzanthracene exhibits a strong fluorescence when dissolved in »Carbowax» (12), or solubilized in the aqueous solutions of association colloids (6, 7, 18).

Some experiments with colchicine were also made. They were carried out in the same manner as reported earlier (15, 17). Colchicine (Merck, Darmstadt) was dissolved in dioxane to give a concentration of 0.1 per cent. The concentration was also the same in the association colloid-group.

The pathological specimens were investigated in usual way.

RESULTS

Fluorescence Studies

The number of animals in the fluorescence experiments was about 75. In an earlier paper (14) (and unpublished) it has been described some observations concerning the general histological distribution of the carcinogen when applied to skin of mice. It appeared that the carrier of the carcinogen seemed to exert some influence upon the general histological localization of the substance in the skin. According to Berenblum and Schoental (1), we found that when acetone and benzene were used as solvents for 9.10-dimethyl-1.2-benzanthracene, a considerable amount of the solvent evaporates immediately, and the effective concentration of the carcinogen is therefore much higher than the original concentration of the solution. Crystalline hydrocarbon was observed at the skin surface. The histological localization and distribution of the carcinogen applied in acetone and dioxane was, also in our experiments, the same as has been previously described in the literature.

When unmelted »Carbowax 1500» was used as carrier for the carcinogen there were no, or only a few, hydrocarbon crystals at the surface of the epidermis [1]. The fluorescent material appeared in all layers of the skin in a few minutes after the application of the carcinogen. It seemed that the carcinogen dissolved in water-soluble »Carbowax 1500» was able to enter the epithelial cells, the intercellular spaces and other cutaneous elements quite freely.

When anhydrous lanolin was used as carrier the following could be observed: No crystalline hydrocarbon was detectable on the skin surface. The fluorescent material was observed in the keratin layer of the epidermis and in the sebaceous elements. No certain fluorescent substance, however, was observed in the epidermis itself nor in the subcutaneous fatty layers one hour after the painting of the

[1] Only when the solution was oversaturated.

skin. The histological localization was thus different from that seen one hour after the application of carcinogen in the »ordinary» fat-solvents (acetone, dioxane). It differed also from the distribution when »Carbowax 1500» was used as carrier.

The distribution and localization of the fluorescent material dissolved in lanolin differed also from that when the aqueous solutions of association colloids were used as solvents for the carcinogenic hydrocarbons. These last-mentioned observations will be published in detail in another connection.

Epidermal Carcinogenesis

Acetone and Dioxane as Solvents. — Nothing new was noticed in the present work when compared with the earlier reports in the literature. All the preliminary changes appeared that follow, in general, the application of an acetone (benzene) and dioxane solutions of carcinogenic hydrocarbons, epilation of the skin, destruction of the sebaceous elements, *etc.* For instance, the epilation was distinct by the 2^{nd} to 3^{rd} week of the experiment. All surviving animals had tumours (innocent and malignant) by the 12^{th} to 15^{th} week (various series) of the experiment. By this time all the animals were clearly epilated. This group compriced 50 mice.

»Carbowax 1500» as Solvent. — There was no general epilation at all. Sometimes, however, a limited weak epilation and the development of tumours appeared simultaneously. All surviving animals had tumours after about 18 weeks from the beginning of the experimental series. (Fig. 1.) This group comprised 35 mice.

Lanolin as Solvent. — The epilation process developed slowly and incompletely. In the case of aqueous lanolin (20 mice) the first animals showed signs indicating epilation by the 8th week of the experiment. All surviving animals had tumours by the 17^{th} week.

In the case of anhydrous lanolin (20 mice) there was no complete epilation at all. All surviving animals had tumours by the 15^{th} week of the experiment. (Fig. 2.)

Aqueous Solutions of the Association Colloids as Solvents. — Aqueous solutions of four different association colloids were used in the present work. — (*a*) in the case of aqueous solutions of the non-ionogenic association colloid »Triton NE» the first signs indicating the beginning of epilation appeared by the 3rd week. However, by the 7^{th} week there was a distinct regrowth of the

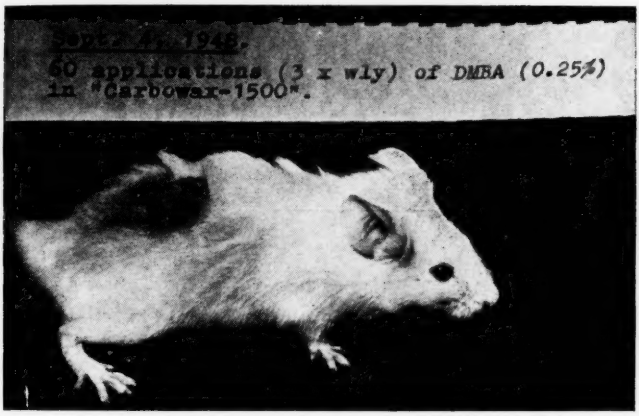

Fig. 1. — Cutaneous tumours induced with 9.10-dimethyl-1.2-benzanthracene dissolved in *water-soluble* »Carbowax 1500». The tumours developed without preceding epilation of the skin.

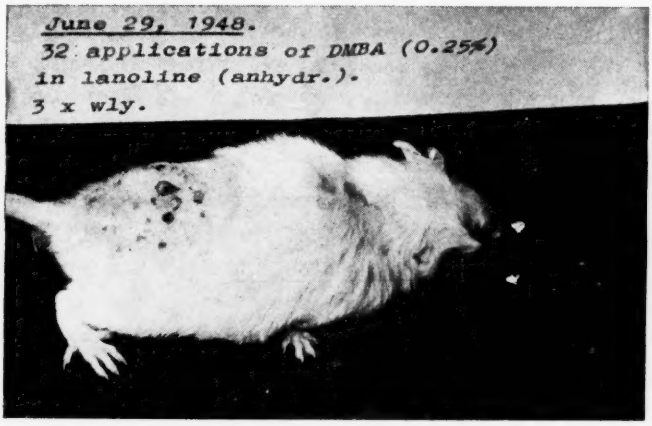

Fig. 2. — Cutaneous tumours induced with 9.10-dimethyl-1.2-benzanthracene dissolved in anhydrous lanolin.

hair in all animals, and after this all the mice were fully haired. Neither was there any distinct epilation in the tumour-bearing animals. All surviving animals had tumours by the 13th week of the experiment. (Fig. 3.) This group comprised 10 mice.

(*b*) in the case of aqueous solutions of sodium cholate the first animals showed a distinct epilation by the 4th week. The majority of the animals showed distinct epilation. All surviving animals had

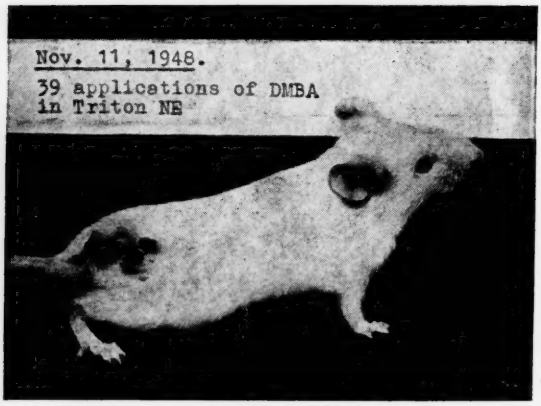

Fig. 3. — Cutaneous tumours induced with 9.10-dimethyl-1.2-benzanthracene solubilized in *aqueous* solution of the association colloid »Triton NE».

tumours after about 13 weeks from the beginning of the experimental series. This group compriced 30 mice.

(*c*) When aqueous solution of sodium myristyl sulphate was used as carrier for the carcinogen, there was a temporarily epilation by the 2nd week of the experiment. But there was a distinct regrowth of hair, and after the 8th week all the mice were fully haired. All surviving animals had tumours by the 19th week. This group comprised 20 mice.

(*d*) When an aqueous solution of sodium oleate was used as carrier for the carcinogen the following could be noted: There were weak signs indicating epilation during the first 3 or 4 weeks. After this the animals were fully haired. All surviving animals had tumours by the 20th week of the experiment. This group comprised 20 mice. (The experiments described in paragraphs *c* and *d* will be published in detail in another connection.)

Some of the results of our experiments are collected in Table 1: the concentration of the carcinogen, evaporation, the presence of crystalline hydrocarbon detected with the fluorescence microscope at the surface of the skin, variation in the »effective concentration», the epilation process (in general), and the »average response» (*i.e.*, the time in weeks of 50 per cent incidence of tumour-bearing animals estimated from the number of mice surviving at the appearance of the first tumour/s/).

TABLE 1

Carrier for the Carcinogen	Concentration (%)	Evaporation	Crystalline Hydrocarbon at the skin Surface	Variation in »Effective Concentration»	Epilation Process (general)	»Average Response» (weeks)
Acetone	0.03	Distinct	Yes	Higher	Distinct	7
Acetone	0.3	Distinct	Yes	Higher	Distinct	6
Dioxane	0.03	Distinct	Yes	Higher	Distinct	7
Dioxane	0.3	Distinct	Yes	Higher	Distinct	6—7
Anh. lanolin ..	0.25	Not evident	No	Unchanged	Weak	10
Aqu. lanolin....	0.25	Not evident	No	Unchanged	Weak	10
Carbowax[2]	0.03	Not evident	No	Unchanged	No(?)	20
Carbowax[2]	0.25	Not evident	No	Unchanged	Weak or No	11
Duponol[1]	0.03	Not evident	No	Unchanged	Weak or No	9
Triton NE[1]	0.07	Not evident	No	Unchanged	Weak or No	9
Sodium cholate[1]	0.03	Not evident	No	Unchanged	Distinct	11
Sodium oleate[1]	0.03	Not evident	No	Unchanged	Weak or No	18

[1] Aqueous solutions of association colloids. [2] In unmelted state.

It has thus been found that (*1*) 9.10-dimethyl-1.2-benzanthracene maintains its carcinogenic activity also when applied in lanolin solution [as reported by Berenblum and Schoental (1)], (*2*) that neither with lanolin nor with »Carbowax« and aqueous association colloids (Triton NE, Duponol, sodium cholate, sodium oleate) was crystalline hydrocarbon observed on the skin surface, (*3*) consequently the »effective concentration» of the hydrocarbon remained thus unchanged in these compounds.

The »Colchicine Effect»

Colchicine has been used in two earlier works (15, 17) as co-carcinogenic agent. It appeared that the effect seemed to be dependent upon several factors modifying the results, *e.g.*, the character of the solvents.

Thus, it appeared that when »Carbowax 1500» was used as carrier for the carcinogen, the average time of response was shortened (when certain concentrations of the carcinogen was used), or the number of individual tumours was increased (15, 17) (and unpublished).

When, however, colchicine (dissolved in dioxane) was applied simultaneously with the carcinogen (dissolved in anhydrous lanolin) the average time of response increased considerably.

A similar observation was made when an aqueous solution of the association colloid »Triton NE» was used as solvent. In some series the addition of colchicine completely inhibited the development of tumours. (These and related observations will be published in detail elsewhere.)

DISCUSSION

Our observations illustrate from different sides the significance of the solvent in connection with skin carcinogenesis in mice. They reveal that the volatility or non-volatility of the solvent used is of considerable importance, but show also that the increase in the concentration of the carcinogen as a result of the evaporation of the solvent is not the only factor to be recognized if one is to clear up the differences between the two groups of solvents studied. Even in each group, for instance, among the non-volatile solvents, marked differences may be noted.

Thus, for instance, fluorescence studies have indicated that the localization of the fluorescent substance shortly after the application depends on what solvent is used. The type of solvent sums to affect the rate by which the carcinogen is absorbed by the skin and the pathways for the passage of the carcinogenic hydrocarbons through the skin. Lanolin solutions differ in this respect, for example, both from the fat-solvent solutions (benzene, acetone, dioxane) and from the non-volatile water-soluble »Carbowax» as well as to some extent from the aqueous solutions of »Triton NE», »Duponol», sodium cholate and sodium oleate.

The first signs (*i.e.*, destruction of the sebaceous elements, changes in the hair follicles, epilation) which precede tumour formation in the skin carcinogenesis in mice when fat-solvents are employed, are partly observed (epilation) also when the carcinogen is applied in aqueous solution of the association colloid sodium cholate, but are not so distinct when lanolin, »Carbowax 1500», aqueous »Triton NE» solution, or the aqueous solution of the association colloid sodium oleate are used as carriers. This indicates that skin tumours (both innocent and malignant ones) can develop without the distinct appearance of the mentioned preceding epidermal changes when certain carriers are employed. Because of this one can ask with good reason whether *these* »precarcinogenic» phenomena

are essential prerequisites in skin carcinogenesis in mice (as earlier supposed).

Experiments with added material (colchicin, 15, 17) substantiate very clearly the observation that the type of solvent is of great importance in chemical carcinogenesis.

The experiments we have described were not conducted with the particular purpose of throwing light on the effective concentration of the carcinogen when volatile and non-volatile solvents are used. Some interesting observations may, however, be mentioned. A concentration of 9.10-dimethyl-1.2-benzanthracene solubilized in aqueous »Triton NE» solution has approximately the same average response as a 3½ times as concentrated solution of the same hydrocarbon in lanolin or in water-soluble »Carbowax 1500». None of these solvents are members of the volatile solvent-group, and hence no change in concentration due to evaporation may be expected when they are used. Further, the average response was the same (from 9 to 11 weeks) with a 0.25 per cent solution of the carcinogen in lanolin and unmelted »Carbowax 1500» as with certain aqueous solutions of other association colloids »Duponol» and sodium cholate containing only 0.03 per cent, *i.e.*, about one eighth as much of the same carcinogen. In these cases there occurred neither evaporation nor variation in the effective concentration at the skin surface.

It is obvious that changes in the concentration occur later in the permeating processes when the carcinogen solution comes in contact with different substances (fluid, semifluid, and solid) on and in the skin. This exchange takes place differently when the solvent and the skin substance in question are wholly or partly soluble in each other or miscible with each other, or when such is not the case. In the latter case we are concerned with the distribution of the carcinogen between solvent and skin substance, and the solubility of the carcinogen in each is the determining factor. It is easily seen that the type and properties of the solvent will to a certain degree determine the way in which the carcinogen solution permeates into the skin elements and which component of the latter comes into contact with the solution in the first place.

The penetration of fluids through the skin is very much restricted, partly because of the keratin covering and the fatty film over the surface, but also because of the close texture of the cells (9, 10, 11). According to a review of this subject by Rothman (10, 11), which will be shortly quoted in this connection, the transitional layer between the cornified and non-cornified epithelium are the real barriers to absorption. He emphasized the importance of an electrical double layer in this region.

The greasy layer consists mainly of waxes and cholesterol esters. It hinders the wetting of the cornified surface and delays the penetration of water and aqueous solutions into the horny layer. Particularly with warm water, the penetration of aqueous solutions into the horny layer becomes possible. The horny layer itself represents a rather rough network of horny lamellae with large holes in it and therefore is easily permeable to ions as well as to large molecules. The barrier at the lower level of the stratum corneum is responsible for the inpermeability of the skin to aqueous solutions of electrolytes.

Qualitatively it has been proved that lipoid-soluble substances penetrate the skin, whereas substances insoluble in lipoids do not. Quantitatively, however, this rule cannot be carried through consistently. Some authors suggest that a certain degree of solubility in water together with high solubility in lipoids is optimum for absorption through the skin. The idea that the permeability to lipoid-soluble substances is due to a sterolphosphatide framework in the cell or in the membrane was reviewed and experimentally supported by Starkenstein and Hendrych (22). According to Rothman (11), any disturbance of the lipoid framework either by precipitation or by dissolution of cholesterol breaks the barrier of skin absorption. Previous treatment of the skin with lipoid solvents (chloroform, ether) which dissolve cholesterol, or with saponins, which precipitate it, makes the skin permeable to water-soluble substances and diminishes the permeation of lipoid-soluble compounds.

The mechanism of percutaneous absorption as a whole becomes rather complicated by the presence in the skin of gland ducts and hair follicles which open on the surface in the form of visible pores. The space in these is loosely filled with greasy, horny scales and air. Any substance may penetrate this space and reach the ducts of

sebaceous glands, and from these the gland cells. This pathway does not require passage through a stratified epithelium but leads through a canal filled with air to cell membranes, the permeability of which is higher than of the transitory epidermal cells. Similarly, the penetration may occur through the sweat gland cells.

Water and aqueous solutions do not enter the follicular and glandular pores by mere capillary attraction because of insufficient wetting. Animal (*e.g.*, lanolin) and vegetable fats and oils penetrate the skin via the pilosebaceous elements (apparatus). Mineral fats and oils also enter the sebaceous glands and are absorbed. Penetration of animal, vegetable and mineral fats through the epidermis proper has never been conclusively demonstrated.

In view of the foregoing it is understable that the solutions of carcinogenic hydrocarbons in various solvents should show a different behaviour. Solutions in fats and oil, lanolin, for example, penetrate the skin through the sebaceous glands. Solutions in the volatile fat solvents (benzene, acetone, dioxane) *also* are drawn into the gland ducts. They destroy the sebaceous elements in mice. These solvents evaporate readily, however, and leave the carcinogen more or less dissolved in the greasy layer of the skin or in the ducts of the sebaceous glands. It would seem most probable that the carcinogen enters the last mentioned way. One should also take into account the possibility that the solvent may cause damage in the sterolphosphatide framework in the epidermis itself and thus effect an alteration of the conditions for absorption in the skin. The water-soluble »Carbowaxes» make it possible for the dissolved carcinogen to enter both through the sebaceous glands and directly through the epidermis. Owing to their low surface tension and definite wetting power also aqueous solutions of the association colloids are able to enter via the sebaceous glands but it may be expected that some of them will be able to enter through the epidermis. Both the aqueous association colloid solutions and the »Carbowaxes» are capable of dissolving cholesterol.

As mentioned above, we have made a systematic search for carriers of carcinogenic hydrocarbons that are »both water- and fat-soluble». The »Carbowax»-type of solvent is the one representative of this kind of solvent that we have met in our studies of chemical carcinogenesis. The aqueous solutions of the various association colloids represent another type. By employing these

new solvents and certain others of various types in connection with certain other factors, we intend to carry out a systematic study of the significance of the solvent in chemical carcinogenesis.

REFERENCES

1. Berenblum, I., and Schoental, R.: Cancer Research 1947:*7:*390.
2. Berg, N. O.: Nordisk Medicin 1948:*40:*1948.
3. Ebbecke, U.: Arch.d.ges. Physiol. 1921:*190:*230.
4. Ekwall, P.: Sjätte Nordiska Kemistmötet, Lund 1947. Berättelse och föredrag, p. 179.
5. Ekwall, P. and Setälä, K.: Acta Chem. Scandinav. 1948:*2:*733.
6. Ekwall, P., and Setälä, K.: Acta Path. Scandinav. 1949:*26:*795.
7. Ekwall, P., and Setälä, K.: Skin Tumours Produced in Mice by Carcinogenic Hydrocarbons Solubilized in Aqueous Solutions of Association Colloids. III (unpublished).
8. Eller, J. J., and Wolff, S.: Arch. Dermat. 1939:*40:*900.
9. MacKenna, R. M. B.: Modern Trends in Dermatology, London 1948.
10. Rothman, S.: Handb. d. normalen u. pathologischen Physiologie. IV:1929, and Suppl. *18:*85:1932.
11. Rothman, S.: J. Lab.clin. Med. 1943:*28:*1305.
12. Setälä, K.: Acta Path. Scandinav. 1949:*26:*223.
13. Setälä, K.: Acta Path. Scandinav. 1949:*26:*280.
14. Setälä, K.: Ann. Med. Exp. Biol. Fenn. 1948:*26:*130.
15. Setälä, K.: Acta Path. Scandinav. 1949:*26:*682.
16. Setälä, K.: Duodecim, 1946:*62:*315.
17. Setälä, K.: Ann. Med. Exp. Biol. Fenn., 1948:*26:*126.
18. Setälä, K., and Ekwall, P.: Acta Path. Scandinav. 1949:*26:*804.
19. Simpson, W. L., Carruthers, C., and Cramer, W.: Cancer Research. 1945:*5:*1.
20. Simpson, W. L., and Cramer, W.: Cancer Research 1943:*3:*515.
21. Simpson, W. L., and Cramer, W.: Cancer Research 1945:*5:*5.
22. Starkenstein, E., and Hendrych, F.: Arch. f. exper. Path. u. Pharmakol. 1936:*182:*664.
23. Twort, C. C., and Twort, J. M.: Lancet 1929:*1:*1108.
24. Twort, C. C., and Twort, J. M.: Ztschr. f. Krebsforsch. 1930:*32:*491.
25. Twort, C. C., and Twort, J. M.: Lancet 1934:*1:*286.

FROM THE UNIVERSITY DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, THE UNIVERSITY DEPARTMENT OF MEDICAL CHEMISTRY, AND THE MARIA HOSPITAL, HELSINKI

NON-SPECIFIC ANTISTREPTOLYSIN REACTIONS IN HEPATITIS SERA

ANTISTREPTOLYSIN TITERS BEFORE AND AFTER REMOVAL OF LIPIDS FROM THE HEPATITIS SERA WITH BENTONITE

By

N. OKER-BLOM, E. NIKKILÄ and T. KALAJA

(Received for publication December 29, 1949)

An apparently non-specific increase in the antistreptolysin (AST) titer occurs in contaminated sera (5, 8) in pleural exudate and in sera treated with acid or alkali (13) and in sera from patients with infectious hepatitis (6, 14, 18). Sievers (14) as well as Westergren (18) has compared the occurrence of these high titers with different liver function tests. Recently Packalen (13) has made an attempt to explain all these non-specific AST reactions in a uniform manner. His opinion is that »the primary mechanism of the non-spesific antistreptolysin reactions is a breaking up of the lipoprotein complexes in which the serum lipids are supposed to occur. The uncovered active-OH groups of the freed cholesterol combine with the streptolysin molecules, thus preventing them from attacking the red cells. The breaking up process may be brought about, *inter alia* by the action of acid or alkali, or bacterial enzymes or enzymes liberated or activated through the disintegration of liver cells in hepatitis or leucocytes present in exudates of some duration».

Packalens experiments show *i.a.* that the AST titer in hepatitis sera becomes normal if the serum lipids are extracted from the

serum for instance with acetone and ether in the cold. According to Blix (1), who has described the method, this does not appreciably change the serum proteins.

Thus the real AST value of a given hepatitis serum could be estimated after extraction of the serum lipids. Serum lipid extraction with acetone and ether according to Blix (1), or with ether at −25° C according to McFarlane (9) would probably be too time-consuming for a serological laboratory. It was concluded that the same result would probably be achieved by using Bentonite for the removol of the lipids. Bentonite (montmorillionite) has been used by Hansen (4) for removal of antiproteolytic serum factors for *i.a.* diphtheria antitoxin purification. Suitable amounts of bentonite sol added to diluted serum also precipitates, besides remnants of fibrin and euglobulin cholesterol and lipids without any appreciable change in the antitoxin content of the serum (4).

It has also been shown (10, unpublished work) that precipitation with bentonite to a given bentonite concentration does not noticeably decrease the serum proteins, while the serum lipids decrease to about 15 per cent of the original amount. It thus seems that the use of bentonite is indicated for the above purpose.[1]

MATERIALS AND METHODS

Materials. — Sera were heated to 56° C. for 30 minutes When not immediately used they were stored at −8 to −15° C.

Methods. — *Antistreptolysin titrations:* The AST determinations were made according to a technique described elsewhere (11). The mean error for AST determinations, in this laboratory, has been calculated to ± 30 per cent (12).

Precipitation with Bentonite Sol. — The bentonite precipitation was performed as follows: Serum was diluted one to ten. According to an unpublished work (10), the differences in total nitrogen and lipids were very small whether the pH of the serum was 5.5 or 7.0 to 7.3 as in normal serum. Therefore the pH was not adjusted before precipitation. The diluted serum was distributed in amounts of 1 cc into Widal tubes. To the first tube was added 1 cc of 0.9 per cent saline, to the following 1 cc of a 0.2 per cent bentonite sol

[1] We are indebted to Dr. A. Hansen, of The State Serum Institute, Copenhagen, who has kindly supplied the bentonite for this experiment.

prepared in distilled water and to each of the following tubes 1 cc of a 0.25 per cent, 0.5 per cent and 0.75 per cent bentonite sol respectively. For the electrophoretic and lipid analysis the precipitation was performed with a 2 per cent bentonite sol added in equal parts to undiluted serum. The tubes were carefully shaken and allowed to stand at room temperature for 15 minutes. After this time there could be seen a very marked flocculation in one or more of the tubes, which were then centrifuged for 10 minutes at 2,000 r.p.m. The supernatant which in the tubes with marked flocculation was water clear, was pipetted off for AST determinations.

Lipid Extraction with Acetone and Ether. — This was performed according to the technique described by Blix (1).

Lipid Determinations. — The method used is one described elsewhere by Vesa and Kalaja (17).

Electrophoretic Analyses. — The electrophoretic analyses were performed with the apparatus described by Tiselius (16) and using an optic system as described by Svensson (15). The serum was subjected to dialysis against a phosphate-NaCl buffer (pH 7.8, ionic strength 0.15) for three days and diluted with the same buffer to a final protein concentration of 1.5 per cent. The electrophoresis was carried out at a temperature of 1.0 C with a potential gradient of 5—6 volts per cm and for 135 minutes. From the diagrams was calculated the mean of the ascending and descending sides for each component and from these relative values the absolute value of each component. The total nitrogen was determined using the micro Kjeldahl method.

RESULTS

The antistreptolysin titer was increased in 29 out of 44 patients with hepatitis, or in 65 per cent of the cases. About 30 per cent of these patients showed arthritic symptoms before or in the beginning of the disease, which were very typical of this epidemic (7). It was not possible to show any correlation between the occurrence of increased AST and arthritic symptoms. The AST titer attained its maximum during the climax of the icteric state, reaching usually normal values during convalescence as soon as the icterus disappeared.

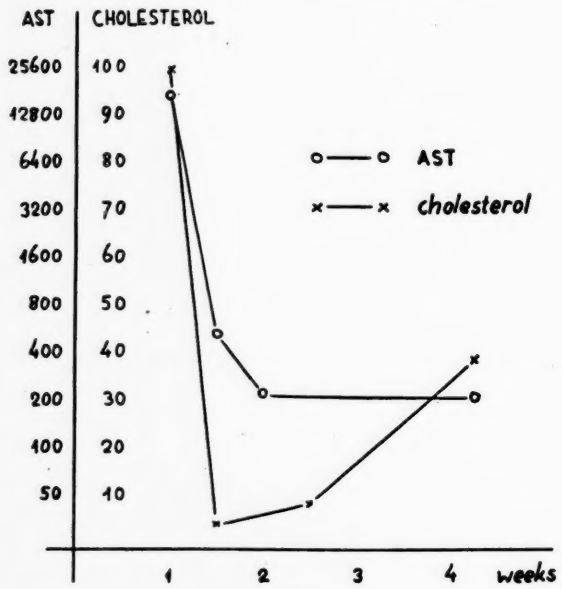

FIG. 1 – AST AND DIFFERENCE BETWEEN TOTAL AND ESTERIFIED CHOLESTEROL IN A PATIENT SUFFERING FROM INFECTIVE HEPATITIS.

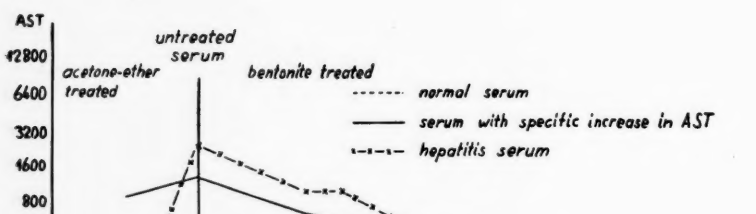

FIG. 2 – EFFECT OF ACETONE-ETHER LIPID EXTRACTION AND BENTONITE LIPID PRECIPITATION ON THE AST TITERS OF NORMALSERA, SERA WITH SPECIFIC INCREASE IN AST AND HEPATITIS SERA.

The correlation between total and esterified cholesterol and AST during the disease in one patient is shown in Fig. 1. During the icteric state there is usually a striking decrease in esterified cholesterol in the serum and a corresponding increase in »free» — *i.e.* not esterified cholesterol (3), and when in this case the amount of »free» cholesterol was at its maximum the AST titer also showed its highest value (Fig. 1).

In Packalens opinion the »free» serum cholesterol inhibits the streptolysin-hemolysis by combining with the streptolysin molecules. In order to prove that the cholesterol really acts upon the streptolysin molecules and does not change the added red cells rendering them less permeable, which probably could be assumed, the following experiment was made. Red sheep cells were suspended in cholesterol suspension of decreasing concentrations starting with a concentration of 0.1 per cent and following twofold dilutions, making a 2 per cent red cell suspension on two series of 12 tubes each. The mixture was incubated at 37° C for half an hour. After centrifugation the cholesterol suspension was pipetted off and in the one series the cells were washed once with 0.8 per cent saline. To all tubes in both series a given amount of streptolysin was added, mixed with the red cells and the tubes were reincubated for 45 minutes longer. A Parallell test was made by mixing the same dilutions of cholesterol (12 tubes) with the same amount of streptolysin incubating the mixture for 30 minutes at 37° C. After the incubation fresh red cells were added and the mixture was again incubated with the other series for 45 minutes more. The results are shown in Table 1.

It is as expected apparent that the inhibition of the hemolysis is

TABLE 1

ANTIHEMOLYTIC EFFECT OF CHOLESTEROL ON RED CELLS AND STREPTOLYSIN

Method	Inhibition of Hemolysis [1]
Cholesterin treated red cells (unwashed) + streptolysin ..	2
Cholesterin treated red cells (washed) + » ..	—
Cholesterin treated streptolysin + red cells	4,096

[1] Inhibition of hemolysis is expressed as reciprocal values of the titer obtained.

due to the action of cholesterol on the streptolysin and not on the red cells.

As mentioned before, precipitation with bentonite sol has been performed using four concentrations of bentonite sol suspensions, and some of the sera were also treated with acetone and ether. Both kinds of lipid extractions have been used in six hepatitis sera, three normal sera and seven sera with apparently a specific increase in AST. Only the results with these sera are shown in Fig. 2. The curves represent the mean of the curves for each serum in the groups.

The lipid extraction *ad modum* Blix gives the same results as given by Packalen, i.e. in sera with specific increase in the AST titer there is only an insignificant decrease in titer whereas the AST titer in hepatitis sera decreases to normal values. In normal sera the circumstances are the same as in the sera with specific rise in AST. After precipitation with the lower concentrations of bentonite sol, i.e. 0.20 and 0.25 per cent the results are nearly similar to those obtained after lipid extraction with ether and acetone. However, the AST titer of hepatitis sera does not decrease significantly with these concentrations. Precipitation with greater concentrations of bentonite sol causes, in all sera, a decrease in AST, which is about proportional to the used concentration of bentonite sol. The decrease is more pronounced in hepatitis sera, and the difference between the behavior of these and the sera with specific AST is statistically significant, but still very small.

According to Hansen (4) an excess of bentonite sol beyond the precipitation limit brings about a loss in antitoxin. Thus it is obvious that the decrease in AST titers after precipitation with the higher concentrations of bentonite sol depends upon an immediate loss in proteins and antibodies which also has been shown elsewhere (10). It could probably be stated in addition that the loss in proteins is somewhat different in different sera and probably most pronounced in hepatitis sera.

Thus the precipitation should be carried out with a bentonite sol concentration not exceeding 0.25 per cent for a serum diluted 1: 10. However, this concentration does not cause any significant decrease in the AST titer of hepatitis sera even if this procedure is expected to decrease the serum lipids considerably. To find the reason the proteins and lipids were analyzed before and after bentonite pre-

TABLE 2

AST DETERMINATIONS, ELECTROPHORETICAL PROTEINANALYSES AND CHEMICAL LIPID ANALYSES IN ONE NORMAL SERUM, ONE SERUM WITH SPECIFIC INCREASE IN AST AND ONE HEPATITIS SERUM BEFORE AND AFTER BENTONITE PRECIPITATION, AND AST DETERMINATIONS AND LIPID ANALYSES IN ONE NORMAL AND ONE HEPATITIS SERUM BEFORE AND AFTER LIPIDEXTRACTION WITH ACETONE-ETHER

Type of Serum	Proteins					Lipids			»AST»
	tot.prot.	alb.	globulins.			tot.chol.	tot. phosph.	ether sol. phosph.	
			α	β	γ				
»Normal» before bentonite prec	7.25	4.07	0.68	0.99	1.51	99	256	244	80
»Normal» after bentonite prec	7.18	4.56	0.36	0.64	1.62	20	81	81	80
»Spec.incr.» before bentonite prec	8.80	3.73	1.02	0.83	3.22	131	230	213	2,500
»Spec.incr.» after bentonite prec	7,22	3.66	0.66	0.38	2.72	10	68	68	1,250
»Hepatitis» before bentonite prec	7.53	3.77	0.75	1.39	1.62	78	369	335	1,250
»Hepatitis» after bentonite prec	5.47	3.75	0.24	0.38	1.10	19	43	37	320
»Normal» before extraction[1]						178	246	218	80
»Normal» after »						34	72	30	80
»Hepatitis» before extraction						178	536	492	6,400
»Hepatitis» after extraction						12	70	20	125

[1] Acetone-ether extraction.

cipitation with three sera electrophoretically and by chemical analysis, and the results were compared with the AST titers before and after precipitation. The results of these analyses are given in Table 2, which also includes the results of similar analyses of one normal serum and one hepatitis serum extracted with ether and acetone.

The electrophoretic analyses show that the mobility of all the serum components before and after precipitation is unaltered.

There is probably a small loss in γ-globulin especially in the hepatitis serum, and in all sera a significant loss in α and β-globulins which is most pronounced in the hepatitis serum; in other words, the bentonite precipitation removes the components of the serum where the greatest part of the serum lipids are supposed to occur (2).

The increase in albumin and total protein in the normal serum is apparently only a fictive one.

The lipid content of all the sera after bentonite precipitation has decreased greatly. For instance, the total amount of cholesterol decreased about 75 per cent. The same holds true also for the phosphatides in the hepatitis serum. It seems as though the remaining phosphatides should belong to the ether soluble phosphatides. The results obtained with ether-acetone extraction of the lipids are similar on the whole. Probably the decrease is more pronounced, but some lipids still remain in the serum. These results are not in full agreement with those of Blix who was able to remove the cholesterol completely but on the other hand according to Blix a certain amount of phospholipids was not extracted which has also been shown here. Probably this depends upon the fact that it was somewhat difficult to keep the temperature at −14° C. The fact still remains that in spite of a similar decrease in lipids the AST titer decreases after acetone-ether extraction, but not after bentonite precipitation.

An attempt was made to compare the bentonite precipitation and ether-acetone extraction in another manner. There is a remarkable increase in AST titer in infected sera. Packalen suggests that the cause is a breaking down of the lipoprotein complexes due to bacterial enzymes. If the lipids are extracted with acetone and ether there is no change in AST whether the serum is infected or not. From the above experiments it seems likely that precipitations with bentonite sol removes in particular these lipoproteins. Thus a serum treated in this way should not show any rise in AST titer after infection. Results confirming this appear in Table 3.

TABLE 3

AST TITERS IN BENTONITE TREATED AND UNTREATED SERA AFTER INFECTION WITH PSEUDOMONAS PYOCYANEUS

Serum	AST		
	Before Infection	After Infection	
		4 Days	6 Days
15809 untreated	100		1,250
treated	64		64
OB11 untreated	250	3,200	16,000
treated	125	200	125

DISCUSSION

Starting from the standpoint that the AST reactions in hepatitis are non-specific the purpose of this work was to find out an easy method for the estimation of the real AST value in these sera. It is apparent that the method used does not adapt it self to this purpose. However, it would seem that these experiments, to some extent, contribute to the question about these non-specific reactions. If Packalens explanation of the primary mechanism for all non-specific AST reactions holds true, which is probable (Table 1, Figs. 1 and 2), the fact that it has not been possible to show any significant difference between the ratio total/esterified cholesterol in normal sera on the one hand and hepatitis sera, infected sera and pleural exudate on the other is surprising. Also it is astonishing that there is no significant decrease in the AST titer in hepatitis sera, despite an approx: 75 per cent decrease in serum lipids following bentonite precipitation. In our opinion there are two possible explanations of these circumstances.

(a) The primary mechanism of the non-specific reactions is that suggested by Packalen, but also lipids other than the cholesterol react with the streptolysin molecules, and probably bentonite does not remove these lipids. Hewitt and Todd (5) have shown that also lecithine inhibits the hemolytic power of the streptolysin although less than the cholesterol. This probably holds true for other serum lipids. Because such lipids occur also in normal sera without any increase in AST, it would seem that the lipids in sera or pleural exudate with non-specific AST have a greater tendency to combine with the streptolysin molecules than »normal» serum lipids.

(b) Some other acetone or ether soluble compounds of the serum, together with the lipids or alone, are responsible for these reactions; possibly compounds which undergo some kind of denaturation during the acetone-ether extraction.

We agree with Packalen that as long as we do not know the exact manner in which the lipids occur in a serum, it is difficult to draw any definite conclusions regarding their part in the non-specific AST reactions.

SUMMARY

Most hepatitis sera show strongly increased AST titers, which apparently are non-specific. About one-third of the patients in this investigation showed arthritic symptoms, which were typical of this epidemic. There was no correlation between the occurrence of arthritis and increased AST.

In one patient the decrease in AST titer from an initial high titer ran parallel to the decrease in free cholesterol.

Precipitations with bentonite sol of concentrations which did not influence the serum proteins had no significant effect on the AST titer in hepatitis sera, although about 75 per cent of the serum lipids were absorbed by the precipitate. On the other hand, extraction of the lipids with acetone-ether resulted in an apparent drop in titer, despite the fact that the residue of lipids was of the same degree as after bentonite precipitation.

The probable explanation of this incongruity is discussed, and it is suggested that the serum lipids in hepatitis sera probably have a greater affinity to the streptolysin molecules than »normal» lipids.

REFERENCES

1. Blix, G.: J. Biol. Chem., 1941:*137:*495.
2. Blix, G., Tiselius, A. and Svensson, H.: J. Biol. Chem., 1941:*137:*485.
3. Greene, C. H., Hatz, R. and Leahy, E.: Archiv. Internat. Med., 1940:*65:*1130.
4. Hansen, A.: Acta Path. et Microbiol. Scand., 1948:*25:*460.
5. Hewitt, L. F. and Todd, E. W.: J. Path. Bact., 1939:*49:*45.
6. Kalbak, K.: The Antistreptolysin Reaction (ASR). I. Technic. Copenhagen, 1947.
7. Klemola, E. and Törmä, S.: Duodecim 1949:*9:*692.
8. Löfgren, S.: Acta Path. Microbiol. Scand.: 1944:*21:*768.
9. McFarlane, A. S.: Nature 1942:*149:*439.
10. Nikkilä, E. and Oker-Blom, N.: to be published.
11. Oker-Blom, N.: Finlands Läkartidn., 1948:*19:*546.
12. Oker-Blom, N.: Ann. Med. Exp. Biol. Fenn. (In press), 1949.
13. Packalen, Th.: J. Bact., 1948:*56:*143.
14. Sievers, O.: Svenska Läkartidn., 1947:*44:*1837.
15. Svensson, H.: Arkiv f. Kemi, Mineralogi och Geologi 22 A.
16. Tiselius, A.: Trans. Faraday Soc., 1937:*33:*524.
17. Vesa, A. and Kalaja, T.: Acta Soc. Med. Fenn. Duodecim 1939:*21:*1.
18. Westergren, A.: Acta path. Microbiol. Scand. 1948:*25:*260.

FROM THE FIRST WOMEN'S CLINIC, UNIVERSITY OF HELSINKI

CHANGES IN THE WHITE BLOOD COUNT AND THE URINARY EXCRETION OF LIPID-SOLUBLE REDUCING SUBSTANCES FOLLOWING RADIUM APPLICATION IN CASES OF CANCER OF THE UTERUS

By

S. PARVIAINEN and P. O. PÄRNÄNEN

(Received for publication January 20, 1950)

There are several clinical methods of estimating the activity of the adrenal cortex. The methods are classified as direct or indirect. The direct methods are based on either biological or chemical analyses of adreno-cortical hormones. Chemical hormone analyses are better suited to clinical practice than the biological ones. Among chemical hormone analyses the method of Heard and Sobel (4) is at present regarded as the simplest and the most practical. It is used for determining the amount of neutral lipid-soluble reducing substances excreted in urine. The reducing property is characteristic of those corticosteroids and their metabolites that are biologically active. The method of Heard and Sobel thus gives a more reliable indication of adreno-cortical activity than does a mere determination of 17-ketosteroids. But all hormone analyses are still relatively complicated. That is why clinical practice has, in general, recourse to the so called indirect methods, by which is meant study of the effects particular to the hormones of the adrenal cortex (13, 14). The simplest one of these methods is observation of the changes in the white blood count.

Selye pointed out that adreno-cortical hormones caused a fall in the lymphocyte count. Dougherty and White (2) found that an injection of the adreno-corticotropic hormone of the hypophysis (ACTH) produced an

increase in the neutrophils and a decrease in the lymphocytes. Forsham *et al.* (3) observed that ACTH caused a considerable fall of circulating eosinophils in patients with normal adreno-cortical function, while patients with Addison's disease failed to show this response. Similar changes in the white blood count have been found to accompany many of the stresses of Selye's general-adaptation-syndrome. A plausible conclusion is that the extent of changes in the white blood count can be used as a simple standard in estimating the reactive property of the adrenal cortex in different individuals.

In view of the above considerations the activity of the adrenal cortex in cancer patients appeared to be a subject worth studying. Parviainen (8) has previously shown that radium therapy is accompanied by quantitative changes in the serum cholesterol and that these changes seem to be indicative of the prognosis of the case. Cholesterol is known to be an important factor in the production of adreno-cortical hormones (12). Thus we have especially wished to elucidate adreno-cortical response to radium application. For that purpose we began to follow changes in the white blood count in cancer patients treated with radium. We have paid special attention to the number of circulating eosinophils, which are considered, e.g. by Thorn *et al.* (13), to decrease the most readily as the hormone production of the adrenal cortex increases.

Isaacson and Rapoport (6) have observed that eosinophilia in a cancer patient is, in the absence of other causes, indicative of dissemination of the malignant process.

Our first aim has been to find out whether the changes in the white blood count that follow radium application may be due to an increased activity of the adrenal cortex. For that purpose we have determined both the white blood count and the urinary neutral lipid-soluble reducing substances in 5 cases.

MATERIAL AND METHODS

Our series consisted of 5 cases, of which 3 were carcinomas of the cervix and 2 were carcinomas of the corpus uteri (Table 1). In all cases white blood counts were made from capillary blood on successive mornings. Simultaneous determinations of the 24-hour urinary excretion of neutral lipid-soluble reducing substances were carried out by the method of Heard and Sobel (cf. Parviainen, Soiva, and Vartiainen (9)).

Table 1 gives the determinations before radium application. It

TABLE 1

Case	Age	Diagnosis
1	40	Ca colli uteri Gr. I (II)
2	30	Ca colli uteri Gr. II
3	55	Ca colli uteri Gr. III (IV)
4	63	Adenoca corp. uteri
5	66	Adenoca corp. uteri

Examinations before Radium Application							
White Blood Count					Total 24-hour Urine Excretion cc	Reducing Substances in Urine	
Number of			Percentage of				
All Leucocytes	Eosinophils	Lymphocytes	Eosinophils	Lymphocytes		mg per 100 cc	mg per 24-hours
10,720	322	2,305	3.0	21.5	1,300	0.201	2.61
6,860	137	2,127	2.0	31.0	710	0.174	1.24
8,000	280	1,680	3.5	21.0	1,100	0.128	1.41
6,400	128	3,168	2.0	49.5	1,250	0.208	2.60
7,000	175	2,135	2.5	30.5	1,380	0.105	1.45

First Radium Application				Prognosis
Radium				
in Uterus mg	in Vagina mg	Time of Treatment hours	Amount of Radium mg hours	
86	80	20	3,320	Bona
86	80	20	3,320	Dubia
—	80	22	1,760	Pessima (cura palliat.)
86	80	20	3,320	Dubia (inoper.)
86	40	20	2,772	Bona (oper.)

shows the number of eosinophils, lymphocytes, and all leucocytes per 1 cmm of blood. The percentages of eosinophils and lymphocytes are given in separate columns. Data are also given of the total 24-hour urine excretion, the amount of reducing substances per 100 cc of urine, and the total 24-hour excretion of reducing substances on the day preceding radium application.

We have selected cases with different prognoses and quantities of radium used with a view to finding out their effect on the excretion of reducing substances and the changes in the white blood

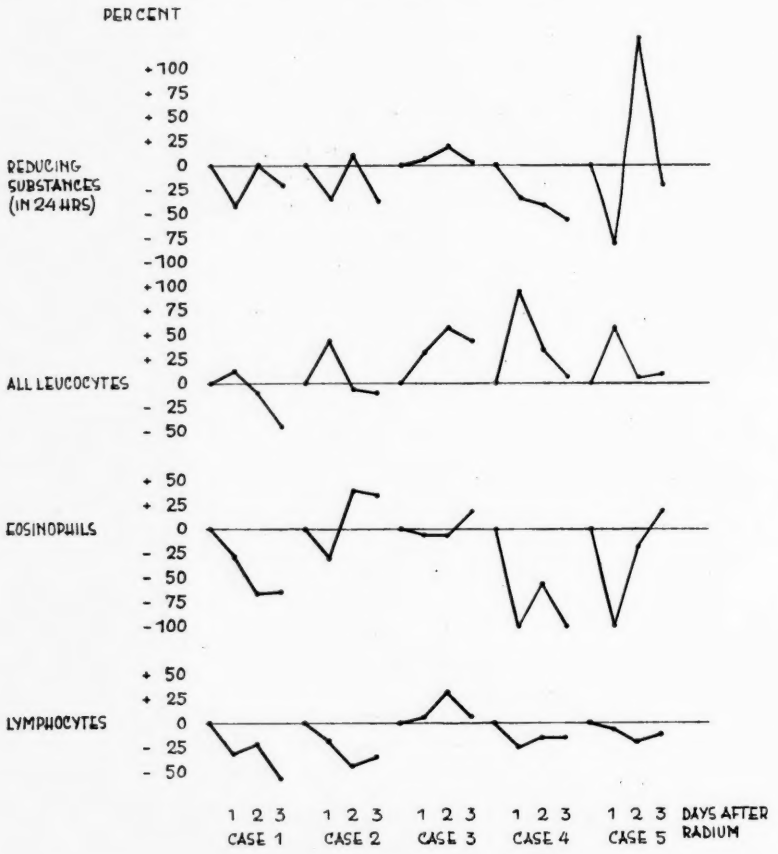

Fig. 1. — Variations in the urinary excretion of reducing substances and in the number of all leucocytes, eosinophils, and lymphocytes following the first radium application.

count. According to the data given in Table 1, the excretion of reducing substances does not seem to be correlated with the white blood count. Thus Case 1 presents the highest amount of reducing substances excreted (2.61 mg per 24 hours), the highest leucocyte and eosinophil counts of our material, and a very high lymphocyte count. In Case 4 again, in which the excretion of reducing substances is as high as in the preceding instance, the leucocyte and eosinophil counts are the lowest, while the lymphocyte count is the highest of our material. The prognosis of this case was extremely unfavourable.

The effect of radium application on the white blood count and the excretion of reducing substances is shown in Fig. 1. The changes are presented with the help of percentage curves. The percentages were calculated according to the method used by Jailer *et al.* (7) with the pre-treatment values as the basis (the first point or 0 per cent). The values for the following days were determined as percentages of the basic value.

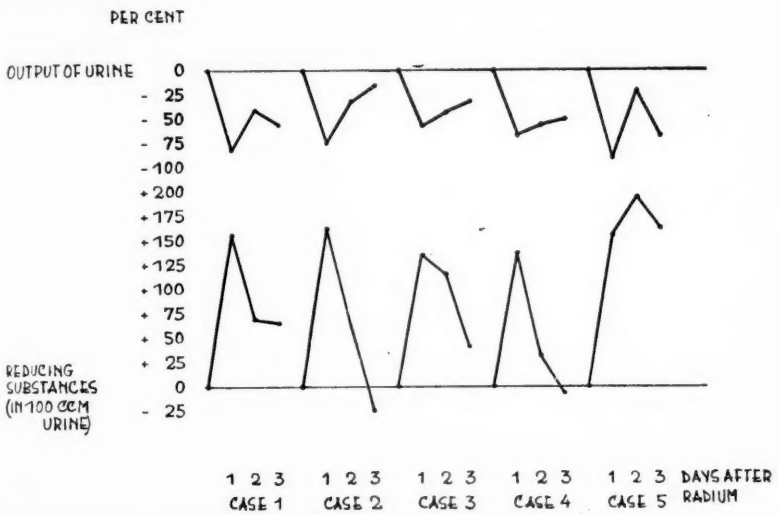

Fig. 2. — Variations in the output of urine and in the concentration of reducing substances in the urine following radium application.

The first curve of each case shows the excretion of reducing substances, the first point being the basis, the second showing how much higher the excretion was on the day on which the patient was treated with radium, the third point giving the percentage on the day following the treatment, and the fourth point giving the value on the second day after treatment. The second, third, and fourth curves give the corresponding percentages of all leucocytes, eosinophils, and lymphocytes before and directly after radium treatment.

As radium therapy, on account of fasting, complete rest in bed, etc., strongly reduces urine excretion, the decrease in urine excretion and the changes in the concentration of reducing substances (mg per 100 cc of urine) are given in Fig. 2. The curves are drawn according to the method used in Fig. 1.

RESULTS

Fig. 1 shows that the excretion of reducing substances decreases during radium therapy. The only exception is Case 3, in which the amount of radium used was the smallest of the material. On the day following the treatment the excretion of reducing substances increased as compared with the amount excreted on the day on which the radium treatment was given. In 3 cases the excretion even exceeded that previous to radium application, the increase being particularly high in Case 5. An exception is Case 4, in which the excretion continued to fall. But clinically this case had the most unfavourable prognosis of the material. On the second day after radium application the excretion of reducing substances decreased in all cases.

According to the above observations the effect of radium application on the excretion of reducing substances varies in different cases. During the treatment the excretion generally seems to decrease. After the treatment there are usually signs of a temporary increase in the excretion.

Great individual variations are also seen in the white blood count. With the exception of Case 3, the leucocyte count rose immediately after radium application and decreased on the following days. The number of eosinophils decreased in all cases directly after treatment, but on the following days the changes varied greatly. The number of lymphocytes also decreased after radium application, except in Case 3.

Examination of the excretion of reducing substances and the changes in the white blood count shows that the adrenal cortex becomes, in general, more active immediately after radium application, as far as these changes can be held to depend on adrenocortical activity alone. Judging from the changes in the white blood count, the activity of the adrenal cortex ought to be considerably more intense than is shown by the excretion of reducing substances. In Cases 3 and 4 the results are contradictory. In Case 4, for instance, the excretion of reducing substances continued to fall, while the changes in the white blood count indicated an increased activity of the adrenal cortex.

As radium therapy is accompanied by a considerable decrease in urine excretion, account has been taken of the changes in urine

excretion and in the concentration of reducing substances (mg per 100 cc of urine). The results are shown in Fig. 2 by curves representing the percentages of the changes. The figure indicates that the concentration of reducing substances greatly rises close after radium application and generally remains higher than the pre-treatment level.

DISCUSSION

The cases studied show that radium therapy, with accompanying fasting, complete rest in bed, etc., produces changes in the total leucocyte, eosinophil, and lymphocyte counts. These changes may be chiefly due to radium, as they were somewhat smaller in the cases in which less radium was used than usual (Cases 3 and 5). The changes, viz. a rise in all leucocytes and a fall in eosinophils and lymphocytes, are such as are claimed to be indicative of an increased adreno-cortical activity. Thus the changes observed in the white blood count and especially in the eosinophil count are likely to indicate the effect of radium application on the activity of the adrenal cortex.

Excretion of reducing substances (mg per 24 hours), which is also regarded as a fairly reliable indicator of adreno-cortical activity, was in 2 of our cases (Cases 3 and 4) contradictory to the changes in the white blood count. In Case 3 the excretion of reducing substances increased together with the number of all leucocytes, while the fall in eosinophils was slight and the lymphocyte count rose.

In this case the change in the lymphocyte count is similar to that seen in psychotic subjects after heat stress (10).

In Case 4 the excretion of reducing substances continued to decrease though the changes in the white blood count indicated an increased production of adreno-cortical hormones. In the two cases (3 and 4) cancer was far advanced and the prognosis unfavourable. It is just in these cases with an unfavourable prognosis that the activity of the adrenal cortex is probably the most seriously disturbed, the contradictory findings being possibly due to the disturbances. As our material is very small, we cannot enter into a detailed discussion of this question, which is further complicated

by several unsolved problems. It should be pointed out, however, that the contradictory changes in the white blood count and the 24-hour excretion of reducing substances may be partly due to radium treatment, which strongly reduces urine excretion. As we have noted in our investigations into toxaemia of late pregnancy (9), the amount of reducing substances excreted in 24 hours by patients with oliguria is not a reliable indication of adreno-cortical activity. In our cancer cases the concentration of reducing substances accords better with the changes in the white blood count than does the total 24-hour excretion of reducing substances.

SUMMARY

The writers have studied the effect of radium application on the activity of the adrenal cortex in patients with cancer of the uterus. In their study they have used the chemical hormone analysis of Heard and Sobel and followed the changes in the white blood count. They have made the following observations: —

During radium application the excretion of reducing substances usually decreases, but it increases temporarily on the day following the treatment. In one case with an unfavourable prognosis the excretion of reducing substances continued to fall.

The number of all leucocytes rises immediately after radium application, while the eosinophils and, in general, the lymphocytes decrease in number. Great individual variations are to be noted on the following days.

Provided that the foregoing changes in the white blood count and the increase in the excretion of reducing substances are in causal relation to the increased activity of the adrenal cortex, the changes observed are sometimes contradictory, particularly in one case with an unfavourable prognosis.

Radium application greatly reduces the excretion of urine and always increases the concentration of reducing substances in the urine.

REFERENCES

1. Davis, M. E., and Hulit, B. E.: J Clin. Endocrinol. 1949:*9:*714.
2. Dougherty, T. F., and White, A.: quoted by Davis and Hulit.
3. Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G.: J. Clin. Endocrinol. 1948:*8:*15.
4. Heard, R. D. H., and Sobel, H.: J. Biol. Chem. 1948:*165:*687.
5. Heard, R. D. H., Sobel, H., and Venning, E. H.: J. Biol. Chem. 1948:*165:*699.
6. Isaacson, N. H., and Rapopert, P.: quoted in 1947 Year Book of Radiology.
7. Jailer, J. W., Marks, D. T., and Marks, P. A.: J. Clin. Endocrinol. 1949:*9:*1074.
8. Parviainen, S., and Kalaja, T.: Acta Soc. Med. Fenn. Duod. Ser. A 1945:*24:*75.
9. Parviainen, S., Soiva, K., and Vartiainen, S.: Acta obstetr. scand. In Press.
10. Pincus, G., and Elmadjian, E.: J. Clin. Endocrinol. 1946:*6:*295.
11. Selye, H.: J. Clin. Endocrinol. 1946:*6:*117.
12. Soffer, L. J.: Diseases of the Adrenals. — Lea & Febiger. Philadelphia, 1948.
13. Thorn, G. W., Forsham, P. H., Recant, L., and Hills, G. A.: J. Clin. Endocrinol. 1948:*8:*589.
14. Tobian, Louis, Jr., and Joseph, H.: J. Clin. Endocrinol. 1949:*9:*677.

FROM THE DEPARTMENT OF PHYSIOLOGY AND THE CHILDREN'S CLINIC,
UNIVERSITY OF HELSINKI

PARALLELISM BETWEEN THE URINARY EXCRETION OF WATER AND AMMONIA

By

O. FRIBERG, M. J. KARVONEN, and V. LEPPÄNEN

(Received for publication January 23, 1950)

It has been stated that the urinary excretion of ammonia has no relation to the water-balance of the body, but that it is conditioned solely by the needs of the acid-base balance of the organism (2). However, when urine samples are collected at relatively short intervals, a distinct — if not absolute — parallelism between diuresis and ammonia excretion has been observed in man (11).

The excretion of sodium and potassium, on the other hand, has been known, since the 19th century, to follow the diuresis (6, 7). The variations in their excretion in the course of diuresis have generally been ascribed to the varying tubular reabsorption of these ions. However, the corresponding parallelism between the excretion of ammonia and water has been suggested a quite different interpretation: The tubular excretion of ammonia would be regulated by the amount of sodium present in the tubular fluid (11).

In the present work, the course of water, ammonia, sodium and potassium excretion as well as that of the urinary pH was followed in unanaesthetized dogs, during water diuresis. The results were interpreted in a way differing from that presented above: suggesting a reabsorption of ammonia along with the tubular reabsorption of water and of other electrolytes.

MATERIAL AND METHODS

Three trained bitches of mixed breed were used for the experiments. Before each experiment, the dog was fasted overnight. In order to bring the animal to a standard state, it was given a pre-experimental hydrating dose of 30 ml of tap water per kg body weight by gavage, 90 min. before the experiment. 30 min. before the experiment the dog was taken to a Pavlov stand and catheterized. A speculum of Reinius (9) (a piece of glass tube, 10 cm long, 1.0 cm in diameter) and a soft, elbowed No. 10 catheter were used. The catheter was left in the urethra for the whole time of the experiment. The urine was collected in graduated cylinders, against the maximum negative pressure of 100 cm H_2O. In nine experiments (3 on each dog), the same amount of water as before was administered at the beginning of the experiment. In six experiments, no second dose of water was given. The animals were trained to this procedure, and did not resent it.

The urine was collected for at least 1½ hrs., or until the diuresis had subsided. Thus, altogether 140 samples of urine were obtained, in which the primary experimental variation was in the excretion of water. In these samples, ammonia was determined by Conway's (2) method, and sodium and potassium with a flame photometer (4). (Two of the samples were too small to allow the determination of sodium and potassium.) The *p*H was determined with a »Radiometer» glass electrode *p*H-meter; if the amount available was very small, indicator paper was used.

For the statistics, current methods were used (8). The correlation coefficients and their standard errors were calculated. The significance of the difference between zero and the observed correlation coefficients was evaluated with the *t*-test. In the tables, *p* denotes the probability that a random sample gives an equal distribution, and *t* the corresponding factor.

RESULTS

The concentration of the electrolytes varied under the experimental conditions within wide limits: Na from 1.10 to 123.0 milliequivalents per litre, K from 0.53 to 23.0 m.eq. per l., and NH_4 from 0.96 to 24.6 m.eq. per l. As compared to the corresponding

normal values in plasma and — accordingly — in the glomerular filtrate, the sodium values in urine were lower, the ammonium values higher, and the potassium values varied on both sides of the plasma concentration, being, however, in general smaller than the normal plasma value.

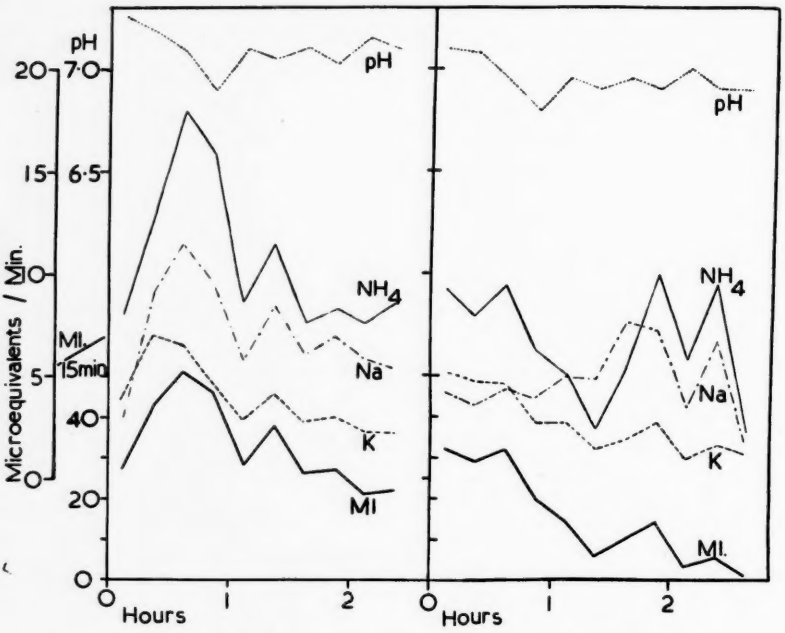

Fig. 1 Fig. 2

Fig. 1. — The results of an experiment on dog called »Corporal», 11.4 kg. At time 0, 342 ml of tap water was given by stomach tube.

Fig. 2. — The results of an experiment on dog called »Diogenes», 11.2 kg. No water was administered during the experiment.

When the excretion of the different components of the urine, the *p*H and the diuresis are plotted against the course of each experiment, it is observed that the excretion of water and that of the cations analysed in general ran parallelly. The *p*H showed relatively slight changes. In ammonia excretion, long-term changes evidently occurred, which tended to keep the level of ammonia excretion approximately constant. These long-term changes appear as having been superimposed to a short-term variation with diuresis. The excretion of sodium also followed diuresis, but at low levels

of diuresis — less than 20 ml per 15 min. — the sodium excretion showed a tendency to rise to a higher level. For potassium, a similar relationship did not obtain, or it was questionable. When the sodium excretion was relatively high, it evidently varied with water diuresis proportionately more than the excretion of the water itself. For ammonia and potassium, the variation appeared proportional to water diuresis. At low levels of diuresis, the parallel course of diuresis and electrolyte excretion was not very obvious.

Two representative examples of individual experiments are given in the form of a graph in Figs. 1 and 2.

The range of variation in the excretion of the various components of the urine is shown in Table 1. Arbitrarily, the excretions obtained with very low urine flows — diuresis less than 10 ml per 15 min. — are treated as a separate group. According to this table, the excretion

TABLE 1

THE EXCRETION OF WATER, AMMONIA, POTASSIUM AND SODIUM, AND THE *p*H: EXTREME VALUES, AVERAGE, AND STANDARD DEVIATION. n = NUMBER OF SAMPLES

	All Samples	Diuresis More than 10 ml/15 min.	Diuresis Less than 10 ml/15 min.
Volume ml/15 min.	1.7 — 110		
	27.3 ± 17.5	31.6 ± 15.8	4.28 ± 2.16
NH_4 microequivalents per min.	0.96 — 24.6		
	10.61 ± 4.28	10.79 ± 4.15	9.67 ± 5.35
pH	6.2 — 7.4		
	6.87 ± 0.31	6.92 ± 0.28	6.58 ± 0.28
n	140	118	22
Na microeq. per min.	1.27 — 67.1		
	10.61 ± 12.38	11.56 ± 13.00	4.95 ± 4.93
K microeq. per min.	0.37 — 34.6		
	4.44 ± 4.85	4.78 ± 5.07	1.94 ± 0.71
n	138	118	20

[1] Microequivalent = 10^{-3} Milliequivalents.

of ammonia would not be significantly affected by water diuresis. The table also shows that the standard deviation of the sodium and potassium values are higher than the averages themselves, i.e. that their distribution is very asymmetric and does not warrant an ordinary statistical treatment.

The distribution of the ammonia and water excretion values — as well as that of the *p*H figures — was symmetrical enough to justify the calculation of correlations. The results of these calculations are presented in Table 2. This table shows that the ammonia values are correlated both to *p*H and to water diuresis; according to this calculation, the effect of *p*H tends to be more marked than that of diuresis.

TABLE 2

THE CORRELATION COEFFICIENTS OF AMMONIA EXCRETION WITH *p*H AND WITH DIURESIS

n = NUMBER OF SAMPLES

		All Samples	Diuresis More than 10 ml/15 min.	Diuresis Less than 10 ml/15 min.
NH_4 — — pH		— 0.46 ± 0.07	— 0.46 ± 0.07	(— 0.24 ± 0.20)
	t	5.69	5.55	1.08
NH_4 — — Diuresis		+ 0.32 ± 0.08	+ 0.35 ± 0.08	(+ 0.29 ± 0.20)
	t	3.90	3.97	1.34
	n	140	118	22
p = 0.05;	*t*	1.977	1.981	2.080
0.01;	*t*	2.612	2.619	2.845
0.001;	*t*	3.362	3.376	3.850

When the individual experiments were compared, it was observed that between the individual experiments, there occurred moderate variations in the excretion of water and ammonia and in the *p*H, but that in the different experiments the level of the sodium and potassium excretion considerably varied. This variation was also responsible for the asymmetric distribution of their values. In order to exclude the effect of variations from experiment to experiment, the excretion values were recalculated in each experiment as a percentage of the first value of that experiment. Such values express the *relative variation within each experiment.* They proved more suitable for a statistical analysis than the direct values, since the attempted standardization of the experimental conditions had not been sufficiently effective as to these electrolytes.

The correlations between these percentage values are shown in Table 3. The table shows that within each experiment, there is a high positive correlation between the excretion of ammonia and potassium. There is also a significant — if less marked — positive

TABLE 3

THE CORRELATION COEFFICIENTS OF AMMONIA EXCRETION AND OF DIURESIS WITH SODIUM AND POTASSIUM EXCRETION AND WITH EACH OTHER, ALL VALUES CALCULATED AS PERCENTAGES FROM THE FIRST VALUE OF EACH EXPERIMENT.

n = NUMBER OF SAMPLES

	All Samples	Diuresis More than 10 ml/15 min.	Diuresis Less than 10 ml/15 min.
NH_4 % — — K %	+ 0.64 ± 0.05	+ 0.63 ± 0.06	(+ 0.23 ± 0.21)
t	9.60	8.75	1.00
NH_4 % — — Na %	+ 0.35 ± 0.07	+ 0.38 ± 0.08	(+ 0.11 ± 0.22)
t	4.38	4.45	0.48
NH_4 % — — Ml %	+ 0.40 ± 0.07	+ 0.32 ± 0.08	(+ 0.27 ± 0.21)
t	5.59	3.68	1.25
K % — — Ml %	+ 0.69 ± 0.04	+ 0.61 ± 0.06	+ 0.44 ± 0.18
t	11.06	8.27	2.14
Na % — — Ml %	+ 0.21 ± 0.08	+ 0.21 ± 0.09	(— 0.02 ± 0.22)
t	2.46	2.30	0.09
n	138	118	20
$p = 0.05$; t	1.978	1.981	2.101
0.01; t	2.612	2.619	2.878
0.001; t	3.363	3.376	3.922

correlation between the excretion of ammonia and water, and between the excretion of ammonia and sodium. When the correlations of the water excretion are considered, it is observed that the highest positive correlation obtains between the excretion of water and potassium, the second highest between water and ammonia, and only a less significant positive correlation between water and sodium.

The above calculations show that the excretion of ammonia varies to some extent with the excretion of water. However, the calculations do not exclude the effects of any long-term changes in the excretion of ammonia, which might occur within the span of each experiment. In fact, a study of the individual experiments shows that the *excretion of ammonia and water during successive periods* most often changes in the same direction, whereas the excretion of ammonia at the beginning and at the end of an experiment may be of approximately the same order, in spite of a great difference in water excretion. Among the 140 samples, the excretion of ammonia and water changed in the same direction between two successive samples in 115 cases, and in opposite

directions in 20 cases. (In 5 cases, either of these values remained unchanged during successive periods of sampling.)

When the *excretion of ammonia and changes in the pH* were compared in a similar way, semiquantitatively, it was observed that there was hardly any correlation between their short-term variations. The excretion of ammonia and the pH changed in a parallel way in 58 cases, and in opposite directions in 68 cases. (In 14 successive samples, either of these values remained unchanged.)

In 105 cases, the excretion of ammonia and potassium moved in the same direction, and in 25 cases contrarily. For ammonia and sodium, the corresponding figures are 99 and 32.

DISCUSSION

The present results clearly indicate that — under the conditions of the experiments — the excretion of ammonia varies to some extent parallelly with diuresis. The interdependence of the excretion of water and ammonia is most marked, when short-term variations are considered. When the excretion during longer periods is studied, this correlation is no more so obvious. Other factors come into play, notably the well known negative correlation between the urinary *p*H and the ammonia excretion. Therefore, this short-term correlation has evidently escaped the attention of most investigators.

Changes in the ammonia production evidently occur with varying speed in different animal species. In man, the maximum NH_4-excretion is reached in about 4 days, whereas in dog, a few hours are sufficient for that (10). Correspondingly, one would expect that in man, a correlation between diuresis and ammonia production could be observed at considerably longer intervals of sampling than necessary in dogs. In fact, the present investigation was stimulated by a finding that diuresis and ammonia excretion — studied in samples of human urine collected at intervals of 8 hours, during ammonium chloride acidosis — showed a tendency to covariation, which, however, was not statistically quite significant in the material (3).

Under the conditions of the present experiments, major variations in water diuresis are known to be due to a varying degree of tubular reabsorption of the glomerular filtrate. Accordingly,

tubular processes obviously are also responsible for variations in the excretion of electrolytes. For sodium and potassium, the following tubular processes must be considered in this connection:

a) Active tubular reabsorption of ions with the reabsorption of water;

b) Active tubular reabsorption of ions independently of the reabsorption of water;

c) Passive diffusion of ions along the gradient from the tubular fluid to the extratubular space — and to blood.

When ammonia is concerned, a fourth tubular process must be added:

d) Tubular excretion (production) of ammonia.

Which of these processes is responsible for the covariation of the excretions of water and ammonia? It could well be thought that *water diuresis would directly affect the site of the ammonia production.* If variations in the reabsorption of water occur in the nephron at the site of ammonia production or proximally from it, the time of the contact of the tubular fluid with the ammonia-producing cells would depend on diuresis. The less the diuresis, the longer the contact of the urine with the cells. Assuming, further, that there is a dynamic chemical equilibrium between the producing cells and the tubular fluid, a positive correlation between diuresis and ammonia excretion would be expected. With a rapid urine flow, ammonia would be carried rapidly away, and thus the equilibrium would be constantly disturbed in favour of ammonia production.

However, this explanation is made less probable by the markedly high positive correlation between the excretion of ammonia and potassium. For potassium, there is no reason to assume such a mechanism of excretion; therefore, the assumption of an equilibrium between the ammonia-producing cells and the tubular fluid also seems less likely.

On similar grounds as above, an argument for the *passive diffusion of ammonia* from nephron to blood could be supported. But again an objection can be made. The excretion of potassium shows a positive correlation with diuresis, independently, whether its concentration is above or below the concentration in plasma. Therefore, diffusion can not be the main factor in effecting the variations of the excretion of potassium. In an analogy, the same can be reasonably assumed to be valid for ammonia.

The first two possibilities remain: *the active reabsorption either with water or independently of it.* On the basis of the present results it can not be judged which of these processes actually occurs. On general grounds, the reabsorption with water perhaps is the most probable alternative.

The very similar behaviour of potassium and ammonia in the tubuli of the kidney is probably a consequence of the general chemical and physical similarity of these two ions. Their effective ionic radii are very close to each other — K^+ 1.33 Å, and NH_4^+ 1.42 Å — whereas e.g. the radius of Na^+ is 0.95 Å (5). The potassium and ammonium ions are also known to have almost identical hydration energies: 95 and 97 k-cal. per mole (1).

At low levels of water excretion, the excretion of all the electrolytes examined is hardly at all correlated with diuresis. This may depend to some extent on the disturbing effect of the dead space: the fluid contained in the urinary tract and in the catheter effects a certain lag in the excretion, which is most clearly manifest at low diuresis levels. However, in addition to this general lack of correlation, the excretion of sodium often increased, when the diuresis subsided. This increase is probably due to the release of the antidiuretic hormone of the posterior pituitary, and has no direct relation to the excretion of water.

When the correlation between diuresis and the sodium excretion is considered, another special feature has to be marked: the changes of sodium are proportionately in excess of those of water and of potassium. This finding is not new; it has been made as early as in the last century (6).

Among the present results, there are no findings supporting the hypothesis of an interdependence between the excretion of ammonia and sodium (11). In fact, the correlation between ammonia and sodium excretion is rather low. Superficially, it might be claimed that at low levels of diuresis, both often show a relative increase, but the lack of any correlation refutes the significance of such a suggestion.

SUMMARY

1. Altogether 140 samples of dog urine were taken at intervals of 15 min. in the course of water diuresis; diuresis, *p*H, and the NH_4, K, and Na excretion were determined. The results were statistically analysed.

2. The excretion of ammonia occurred to some extent parallelly with diuresis (correlation coefficient + 0.32), and it was in an inverse relation to the *p*H (corr. coeff. — 0.46).

3. The parallelism between diuresis and ammonia excretion was most marked, when short-term changes were considered.

4. There was a high positive correlation between the excretion of ammonia and that of potassium: + 0.64.

5. The results were interpreted as suggesting a tubular reabsorption of ammonia — similar to that of potassium — probably with the reabsorption of water.[1]

REFERENCES

1. BUTLER, J. A. V.: Chemical Thermodynamics. 4th ed. 1949. P. 494. MacMillan and Co. London.
2. CONWAY, E. J.: Micro-diffusion Analysis and Volumetric Error. 1947. P. 98. Crosby Lockwood & Son. London.
3. FRIBERG, O., K. GRÜNN, M. J. KARVONEN, and MAIJA SULAMAA: Unpublished results.
4. HALLMAN, N., and V. LEPPÄNEN: Suomen Kemistilehti 1949:*B 22:*55.
5. Handbook of Chemistry and Physics. 29th ed. 1945. P. 2582. Chemical Rubber Publishing Co., Cleveland, Ohio.
6. KATSUYAMA, K.: Hoppe-Seyl. Z. physiol. Chem. 1899:*28:*587.
7. KATSUYAMA, K.: Hoppe-Seyl. Z. physiol. Chem. 1901:*32:*235.
8. LINDER, A.: Statistische Methoden für Naturwissenschafter, Mediziner und Ingenieure. 1945. Birkhäuser. Basel.
9. REINIUS, L. R. R.: Personal communication.
10. RYBERG, C.: Acta Physiol. Scand. 1948:*15:*114.
11. RYBERG, C.: Acta Physiol. Scand. 1948:*15:*161.

[1] The writers are very grateful to Miss Eila Ala-Ketola, who helped in all the experimental work and performed the ammonia analyses. Two of the writers (M. J. K. and V. L.) have been aided by a personal grant from »Lastentautien Tutkimussäätiö» (Foundation for the Investigation of Children's Diseases).

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

THE RÔLE OF A SO-CALLED AGGLUTINATION ACTIVATING FACTOR IN THE AGGLUTINATION OF HEMOLYTIC STREPTOCOCCI BY RHEUMATOID ARTHRITIS SERA[1]

By

ODD A. WAGER

(Received for publication March 3, 1950)

It has been found that the sera of persons with rheumatoid arthritis are capable of agglutinating hemolytic streptococci in high titres. This phenomenon was formerly considered to be entirely specific, a true antigen-antibody reaction, as the Widal test is in typhoid fever, and it was held to be evidence supporting the theory that infection by *streptococcus haemolyticus* is the etiological factor in rheumatoid arthritis. Several reports describing recent investigations, however, advance the opinion that the streptococcal agglutination reaction in rheumatoid arthritis may be ascribed to some non-specific changes in the patient's serum. Wallis (7) attributes it to a non-specific factor in these sera by which the action of the weak streptococcal agglutinins which already normally are present in the human serum is intensified without increasing the amount of streptococcal agglutinins in rheumatoid arthritis sera.

The sera of patients with rheumatoid arthritis have also been found to possess hemagglutinating properties of a special nature (2). Most human sera agglutinate the red cells of sheep to some degree, but the highest serum dilution at which agglutination occurs does not usually exceed 1: 64. If a suspension of sheep red cells to which a small amount of homologous amboceptor has been added is used

[1] Preliminary report of a study on agglutination conditions in rheumatoid arthritis.

as the antigen, all human sera generally agglutinate such sensitised red cells somewhat more strongly than non-sensitised red cells. With rheumatoid arthritis sera the differential agglutination titre between the sensitised and the non-sensitised red cells (the Rose test) is in most cases more than 16-fold, but with other sera less. If, for instance the highest agglutinating serum dilution for sensitised red cells is 1: 2048 and for non-sensitised cells 1: 32, the Rose test value is $\frac{2048}{32} = 64$. It has been held that also this phenomenon supports the opinion that rheumatoid arthritis sera contain a non-specific factor which activates the agglutination between red cells and the amboceptor used for their sensitisation and is called the agglutination activating factor (4, 5). The Rose test would accordingly demonstrate this factor, for the greater the amount of the agglutination activating factor, the higher the Rose test value. A similar phenomenon is seen with guinea pig, chicken or human red cells sensitised by their respective homologous amboceptor (6). The serum of a patient with rheumatoid arthritis is also capable of agglutinating his own sensitised red cells in high titres (5).

The object of the investigation described below was to study the rôle possibly played by the assumed agglutination activating factor in the agglutination of hemolytic streptococci by the sera of patients with rheumatoid arthritis. The following working hypothesis was assumed: If the serum under test is absorbed with hemolytic streptococci the true streptococcal agglutinins are removed. The agglutination activating factor will remain in the rheumatoid arthritis serum but in the absence of streptococcal agglutinins it does not produce agglutination. Prior to the performance of a fresh agglutination test with the absorbed serum the streptococci are sensitised by adding to the streptococcal antigen a small amount of known streptococcal immune serum. Agglutination of the streptococci can now be expected to occur if an agglutination activating factor is present in the absorbed serum.

EXPERIMENTS

Ten sera were selected for the tests (Table 1). The streptococcal agglutination titre and the Rose test value were determined for each serum, employing the general principle of Rose's technique (2) for the latter and Thulin's technique (3) for the streptococcal

agglutination and the absorption tests. The streptococcal antigen was *streptococcus haemolyticus* strain S.F. 130 employed by Kalbak (1) in routine determinations of streptococcal agglutinins. The immune serum was prepared by immunising rabbit No. 52 with the same streptococcal strain.

In table 1 the sera are divided into the following five groups:

I. Clinically typical cases of rheumatoid arthritis;
II. A clinically uncertain borderline case between rheumatic fever and rheumatoid arthritis;
III. Cases with a possible etiology of streptococcal infection;
IV. Normal control from a healthy person;
V. Serum from a rabbit immunised with streptococci.

TABLE 1

DIAGNOSES, STREPTOCOCCAL AGGLUTINATION TITRES AND ROSE TEST VALUES OF 10 SERA

Group	Serum	Clinical Diagnosis	Streptoc. Aggl. Titre	Rose Test
I	O.S.	Rheumatoid arthritis	1: 320	2048
	S.M.	—»—	1: 640	1024
	A.R.	—»—	1: 320	512
	E.P.	—»—	1: 320	64
II	V.L.	Rheumatic fever? Rheumatoid arthritis?	1: 640	512
III	O.R.	Abscessus peritonsillaris	+1: 80±1: 160	8
	E.S.	Nephritis acuta	+1: 80±1: 160	4
	A.S.	Tonsillitis chronica Carditis? Insuff. cordis Fibrillatio atriorum Bronchitis	1: 640	4
IV	O.W.	Healthy	1: 20	2
V	Rabbit No. 52	Immunised with streptococcus haemolyticus strain S.F. 130	1: 2560	1

Main Test. — The sera under test, with the exception of that of the healthy person (O.W.), were absorbed with streptococci. The following tests were then carried out simultaneously with absorbed and non-absorbed sera:

a) Determination of the agglutination titre of the serum for non-sensitised streptococci (Table 2, points 1 and 5);

b) Determination of the agglutination titre of the serum for sensitised streptococci (Table 2, points 2, 3, 4, 6, 7, 8 and 9).

RESULTS

The test results listed in table 2 show that the agglutination of the streptococci by non-absorbed sera apparently does not depend on whether the streptococci are sensitised or not (Table 2, points 1—4). This is not the case, however, with sera which had been absorbed with streptococci, for not a single one of these sera agglutinated non-sensitised streptococci in the lowest serum dilution employed, i.e. 1: 20 (Table 2, point 5). On the other hand, sensitised streptococci were agglutinated by the sera in groups I and II, and the agglutination titre was the higher, the greater the dose of immune serum used for sensitisation of the streptococci (Table 2, points 6, 7, 8 and 9). The absorbed sera in groups III and V did not agglutinate sensitised streptococci (Table 2, points 6, 7, 8 and 9). It also deserves note that all the sera which agglutinated sensitised streptococci after absorption had a high Rose test value, and those that did not had a low value.

DISCUSSION

It has been demonstrated in these tests that rheumatoid arthritis sera absorbed with streptococci agglutinate sensitised streptococci and that the agglutination titre is directly proportional to the dosage of immune serum used for sensitisation. This phenomenon cannot be ascribed to streptococcal agglutinins which possibly may remain in the serum after absorption nor to the agglutinins in the immune serum used for sensitisation of the antigen. Even together these are too weak to produce agglutination. This possibility is also convincingly counterargued by the fact that no agglutination of sensitised streptococci was produced by the absorbed streptococcal immune serum nor by the absorbed sera in group III. The most probable explanation is the presence in rheumatoid arthritis sera of an agglutination activating factor which activates the specific agglutination between streptococci and the immune serum used for sensitisation of the streptococci. It is apparent that this factor is identical with that which in the Rose test activates

TABLE 2

AGGLUTINATION TESTS WITH ABSORBED AND NON-ABSORBED SERA, USING SENSITISED AND NON-SENSITISED STREPTOCOCCI AS ANTIGEN

	Immune Serum Dose Used for Sensitisation of Streptococci	Sera									
		I				II	III			IV	V
		O.S.	S.M.	A.R.	E.P.	V.L.	O.R.	E.S.	A.S.	O.W.	Rabbit 52
		Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre	Str. Aggl. Titre
with non-absorbed sera	1) —	1: 320	1: 640	1: 320	1: 320	1: 640	+1: 80 ±1: 160	+1: 80 ±1: 160	1: 640	—	1: 2560
	2) ½ × minimum agglutinat. dose	1: 320		+1: 320 ±1: 640	1: 640	1: 640			1: 640	—	1: 1280
	3) ¼ × » » »	1: 320		1: 320	1: 320	1: 640	1: 80		+1: 320 ±1: 640	—	1: 1280
	4) 1/8 × » » »	1: 320		1: 320	1: 320	1: 640			1: 640		1: 1280
with absorbed sera	5) —	—	—	—	—	—	—	—	—		—
	6) ½ × minimum agglutinat. dose	1: 320	1: 1280	+1: 40 ±1: 80	1: 640	1: 160	—	—	—		—
	7) ¼ × » » »	1: 80	+1: 160 ±1: 320	1: 40	+1: 320 ±1: 640	1: 160	—	—	—		—
	8) 1/8 × » » »	1: 40	1: 160	±1: 40	1: 160	1: 160		—	—		—
	9) 1/16 × » » »	±1: 40		—	+1: 40 ±1: 80	1: 80		—	—		—
	Rose Test	2048	1024	512	64	512	8	4	4	2	1

the specific agglutination between sheep red cells and the homologous amboseptor. This explanation is borne out also by the correlation which in the tests now made can be noted between the Rose test values on the one hand, and the agglutination of sensitised streptococci on the other hand.

CONCLUSIONS

1. The agglutination of hemolytic streptococci by sera from patients with rheumatoid arthritis apparently is not due to a supposedly greater amount of specific streptococcal agglutinins in these sera in comparison with other sera.

2. The agglutination of streptococci by rheumatoid arthritis sera cannot be held to be evidence in support of the theory that a *streptococcus haemolyticus* infection is the etiological factor in rheumatoid arthritis.

SUMMARY

It was the object of this work to study the rôle possibly played by a so-called agglutination activating factor in the agglutination of streptococci by sera from patients with rheumatoid arthritis. Tests involving the examination of ten different sera showed that rheumatoid arthritis sera absorbed with streptococci agglutinated sensitised streptococci. It is regarded as probable that this is produced by an agglutination activating factor present in rheumatoid arthritis sera. It is therefore the author's opinion that rheumatoid arthritis sera do not contain more specific streptococcal agglutinins than do other sera, and that the agglutination of hemolytic streptococci by rheumatoid arthritis sera does not constitute evidence in support of the theory of the streptococcal etiology of rheumatoid arthritis.

REFERENCES

1. KALBAK, K.: Nord. Med. 1946:*31:*1997.
2. ROSE, H. M., RAGAN, C., PEARCE, E., & LIPMAN, M. O.: Proc. Soc. Exp. Biol. and Med. 1948:*68:*1.
3. THULIN, K. E.: Serological Aspects of Hemolytic Streptococci. Academic Thesis. Lund 1948.
4. WAALER, E.: Acta Path. Microbiol. Scand. 1940:*17:*172.
5. WAGER, O.: Ann. Med. Exp. Biol. Fenn. 1950 (in press).
6. WAGER, O.: Unpublished data.
7. WALLIS, A. D.: Am. J. Med. Sci. 1947:*213:*94.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

NATURE OF HEMAGGLUTININS IN SEEDS OF CYTISUS SESSILIFOLIUS

By

ROLF KOULUMIES

(Received for publication March 14, 1950)

A saline extract prepared from seeds of *Cytisus sessilifolius* agglutinates chiefly O and A_2 red cells (14). It was found that this seed extract can be used for the differentiation of A red cells into subgroups A_1 and A_2 (7). Experiments made with a purified product of this seed extract will here be described.

PURIFICATION OF THE SEED EXTRACT OF CYTISUS SESSILIFOLIUS (15)

The seeds were ground to a fine powder in a mortar. The powder was extracted with saline in the weight proportion of 1 : 10, incubated for 2 hours at 37° C and centrifugated at approx. 3 000 r.p.m. The supernatant was then used as cytisus extract, to which one-fourth by volume of 94 per cent alcohol was added and the extract held for 24 hours at room temperature. It was then centrifugated, the precipitate removed, and one-half by volume of acetone was added to the supernatant. After standing for half an hour it was centrifugated and the precipitate collected. The precipitate was diluted in the original volume of saline, one-half volume of chloroform was added, and the substance was shaken for one hour in a shaker and centrifugated. Three layers formed and the top one was collected, precipitated with a double volume of acetone, and

TABLE 1

Red Cell Group	Dilution of Crude Cytisus Extract							Dilution of Purified Cytisus Extract							
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12800	1 : 25600	1 : 51200	1 : 102400
A_1	—	—	—	—	—	—	—	tr	tr	—	—	—	—	—	—	—
A_2	3	3	3	2	1	—	—	3	3	3	3	2	1	tr	—	—
O	3	3	3	2	1	tr	—	3	3	3	3	3	2	1	tr	—
B	—	—	—	—	—	—	—	2	2	1	1	tr	—	—	—	—
A_1B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Strength of Agglutination: — No Agglutination
tr indefinate or extreamely weak aggl.
1 weak agglutination
2 definit agglutination
3 strong agglutination

The reaction was made in small (10 × 75 mm) test tubes. Amount of reagents: extract dilution 0.1 ml and 1% blood cell suspension 0.1 ml. The results were read by the naked eye after the test tubes had been kept at room temperature (20 C) for 60 minutes.

left standing at room temperature for half an hour, after which it was centrifugated and the precipitate obtained was solved in one-fifth of the original volume of saline. It was re-shaken with chloroform, centrifugated and precipitated with acetone, after which it was centrifugated and the precipitate solved in one-tenth of the original volume of saline. It was shaken once more with chloroform as above, precipitated with acetone, centrifugated, and the precipitate solved in distilled water. It was then again precipitated with acetone and the precipitate obtained was washed with ether and dried quickly in an exsiccator.

Out of 20 grams of seed powder 44 mg of water-soluble fine white powder was obtained. The opalescent fluid, of which a small insoluble part was extracted by centrifugation at 3,000 r.p.m., was used for the agglutination tests. The dried powder stored in the refrigator at + 4° C was found to have maintained its titre for agglutinating O red cells stable even after three months.

The agglutination experiments set out in table 1 were carried out with the original crude cytisus extract and with the purified

extract. The concentration of the extracts is stated as the ratio between the weight of the powder and the volume of the saline used for dilutions.

As will be seen from table 1, the titre of the cytisus extract for O and A_2 red cells was increased approx. 100-fold by means of the described purification. No agglutination was produced in A_1B cells by the dilution used. The A_1 cells showed indefinite agglutination (tr) in the first two tubes, whereas with the B cells the titre of the purified cytisus extract was considerably higher than that of the crude extract. In earlier titration tests with B red cells using crude cytisus extract no reaction had been observed (20 blood samples tested).

The amount of purified cytisus powder obtainable was, however, so small that it was impossible to test enough B red cells to prove statistically whether there are such differences in the agglutination titre of the red cells of different B persons as would be assumed, if a strong reaction of the B blood is caused by heterozygous B (BO) blood. The following experiment was therefore made:

REACTIONS OF PURIFIED CYTISUS EXTRACT WITH GENOTYPICALLY KNOWN BLOOD CELLS

Such married persons as belonged to the AB group were collected from the local blood donor service, and the blood groups of the couples were determined. From these the AB × AB and the AB × O couples were chosen whose children's genotypes are known according to Bernstein and Thomsen et al. (1, 17). There was naturally the possibility of illegimate cases, but all the children in these families were examined and they fitted all in with the above mentioned theories. Agglutinationslide-tests were made using an anti-O cattle serum absorbed with A_1B blood cells, in the dilution 1: 2. Blood cells Nos 1), 2), 8) and 12) gave strong reactions, whereas the other tests were completely negative with this serum. The blood samples were titrated with the purified cytisus extract and the results are shown in table 2.

As is seen from table 2, the purified cytisus extract agglutinated O and A_2 cells very strongly, with results comparable to those

TABLE 2

Red Cell Group				Dilution of Purified Cytisus Extract								
No.	Code	Phenotype	Genotype	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12800	1 : 25600	1 : 51200
1	(M.K.)	O	OO	3	3	3	3	3	2	1	1	—
2	(M.T.)	A_2	?	3	3	3	3	3	2	1	tr	—
3	(R.K.)	A_1B	A_1B	1	tr	tr	tr	—	—	—	—	—
4	(T.S.)	B	BB	1	1	tr	—	—	—	—	—	—
5	(K.H:maa)	A_1	A_1O	—	—	—	—	—	—	—	—	—
6	(Liisa K:ja)	A_2B	A_2B	1	1	1	1	tr	—	—	—	—
7	(Liisa K:nen) ..	A_1	A_1A_2	1	1	1	tr	—	—	—	—	—
8	(T.K.)	B	BO	2	2	2	2	1	1	tr	—	—
9	(H.K.)	A_1	A_1O	1	1	tr	tr	—	—	—	—	—
10	(KePi)	A_1B	A_1B	1	1	tr	tr	—	—	—	—	—
11	(TePi)	B	BB	1	1	tr	tr	—	—	—	—	—
12	(KaPe)	O	OO	3	3	3	3	3	3	2	1	—

Technique and number system for strength of agglutination as in Table 1

shown in table 1. The purified cytisus extract agglutinated BO red cells fairly strongly, thus differing from the agglutination reaction of BB red cells with regard to both strength of reaction and titre. The agglutination of A_1O, A_1B, A_2B and BB blood cells were weaker than those mentioned above. One of the two A_1O blood samples was even completely negative in the lowest dilution used. The limited material does not give the right to draw more far-reaching conclusions.

AGGLUTINATION INHIBITION TESTS WITH PURIFIED CYTISUS EXTRACT AND WITH SECRETOR AND NON-SECRETOR SALIVA

The secretor or non-secretor qualities of the persons used in this test were determined earlier several times and on every occasion they appeared to be the same and the inhibition titres remained on approximately the same level. Previously reported (13, 18, 20) observations were taken into account in the technique: The saliva was collected into a centrifuge tube, avoiding nasal mucus and

TABLE 3

Saliva			Cattle Serum							Purified Cytisus Extract						
			Saliva Dilution							Saliva Dilution						
No.	Blood Group	Secretor Group	1 : 10	1 : 30	1 : 90	1 : 270	1 : 810	1 : 2430	Kontr.	1 : 10	1 : 30	1 : 90	1 : 270	1 : 810	1 : 2430	Kontr.
1	O	S	—	tr	tr	1	2	2	2	—	—	—	—	2	2	2
2	O	S	—	—	—	—	1	2		—	—	tr	1	2	2	
3	O	s	2	2	2	2	2	2		2	2	2	2	2	2	
4	O	S	—	—	tr	1	2	2		—	—	—	1	2	2	
5	O	S	—	—	tr	1	2	2		—	—	—	tr	1	2	
6	O	S	—	—	tr	1	1	2		—	—	—	1	1	2	
7	O	S	—	—	tr	1	2	2		—	—	1	2	2	2	
8	O	s	2	2	2	2	2	2		2	2	2	2	2	2	
9	B	S	—	—	1	2	2	2		—	—	1	2	2	2	
10	A_1B	S	—	tr	tr	1	2	2		—	—	1	2	2	2	
11	A_1	S	—	—	tr	1	2	2		—	—	1	2	2	2	
12	A_1	s	2	2	2	2	2	2		2	2	2	2	2	2	

S = secretor
s = non-secretor

Strength of agglutination as in Table 1.

sputum. Reddish saliva were discarded. Immediately after collection, the saliva was boiled in a water bath for 10 minutes and centrifugated at once at 3,500 r.p.m. The bluish opalescent supernatant was drawn with a pipette into a steril test tube and stored in the refrigator. Changing the pipette, the saliva was diluted with saline. Dilutions stronger than 1: 10 were not used in order to eliminate non-specific inhibition. The strength of the agglutinating serum (or extract) used in all tests was 8 times the agglutinating dose. By means of a pipette 0.1 ml of saliva dilution and 0.1 ml of agglutinating serum (resp. extract) were drawn into a small (10 × 75 mm) test tube, stirred, and allowed 60 minutes to incubate, after which 0.2 ml of 1% 0 red cell suspension was added. The reaction was read by the naked eye 90 minutes after addition of the blood cell suspension. The results are shown in table 3.

As seen in table 3, O-secretor saliva inhibited the agglutinating action of purified cytisus extract on 0 red cells. However, the inhi-

bition titres were low. In series of tests made with cattle serum the lowest titre was 1: 10 and the highest 1: 270; correspondingly with cytisus extracts 1: 30 and 1 : 270. The saliva of three secretors tested in groups B, A_1B and A_1 also inhibited the agglutinating action of cattle serum and cytisus extract on O-cells. Also in these cases the inhibition titres were low, although they were much higher when the same cases were tested with human serum (using always the same 8 — fold agglutinating dose): in case No. 9 (blood group B) = 1 : 2 048, case No. 10 (A_1B) = 1: 80 and case No. 11 (A_1) = 1 : 1 024. With cattle serum the inhibition titres in the corresponding cases were 1: 30, 1 : 10, and 1 : 30 and with cytisus extract 1: 30 in all the cases. Thus the observation made, corresponded to that reported earlier (20). The O and A_1 non-secretor salivas tested did not inhibit the agglutinative action of cattle serum or cytisus extract on O red cells.

DISCUSSION

The Bernstein theory of isoagglutination blood groups and their heredity, which presupposes the presence of the O gene, has prompted investigators to search for the anti-O agglutinins. These have been found in rare cases in human sera belonging to the A_1 and A_1B groups (5, 8, 9). They have also been produced by isoimmunisation, i.e. immunisation of A_1B persons with O cells (19). Anti-O agglutinins have also been frequently found in normal cattle serum (16). In fact, cattle serum from which the A and B agglutinins have been eliminated by absorption with A_1B cells, is the most widely used anti-O agglutinin because of its easy availability. Powerful anti-O sera have been obtained by immunisation of goats with strains of *Shigella dysenteriae* (3). Sera containing anti-O agglutinins have also been produced by immunisation of rabbits with a purified preparation of O-substance isolated from the pseudomucinous ovarian cyst fluid obtained from a person belonging to group O (10). Serum from the eel (*Anguilla anguilla*) has also been found to contain anti-O agglutinins (6). Numerous other animal and human sera containing anti O agglutinins have been obtained either naturally or by immunisation (4), but those mentioned above are the most important. As far as is known to me,

reagents of vegetable origin containing anti-O agglutinins had not been found before Renkonen (14) discovered them in the seeds of the *Cytisus sessilifolius*.

It has been claimed with regard to the above mentioned so-called anti-O sera that, with the exception of the anti-O agglutinins found in some human sera, they are not true anti-O sera, in other words do not extract O genes in red cells but a heterogenetic antigen of the Forsman antigen type, a so-called H-substance, and that they should therefore be called anti-H agglutinins (2, 11, 12). This H-substance would be common also for blood cells other than the O cells. Anti-O and anti-H agglutinins would thus have the common property that they agglutinate predominantly O and A_2 red cells. The agglutinating action of the anti-H agglutinins on O cells is inhibited by the H substance, which is found e.g. in the saliva of O secretors (and also of secretors of other blood groups) and in the pig's gastric mucin, whereas the H substance does not inhibit the agglutinating action of the true anti-O agglutinin on O cells (11).

In the tests described above the agglutinating action of the purified cytisus extract has been studied, and it can be stated that this extract agglutinated predominantly O and A_2 red cells. The agglutinating action of the cytisus extract on 0 cells was inhibited by the saliva of the O secretors (and probably also of secretors belonging to other blood groups). It would therefore seem that the haemagglutinin present in cytisus extract probably is an anti-H agglutinin. Because of the limited test material used this can not be definately claimed, but in any case the characteristics which indicate the anti-H action of the cytisus extract are interesting enough to justify continued studies.

SUMMARY

1) By purification of the saline extract of seeds of *Cytisus sessilifolius* according to the procedure described, a reagent was obtained, which had an agglutinating power on O cells rising to about 100 times that of the original. At the same time the agglutination titre of the reagent for B cells and to some extent also the agglutination titre for other red cells was increased. The reaction

of genotype BO blood was stronger than that of genotype BB which latter was as weak as the reactions of A_1A_2, A_1O, A_1B and A_2B red cells.

2) The agglutinating action of the cytisus extract in question on O-cells was inhibited by secretor saliva (and possibly also by secretor saliva of other groups). The agglutination was not inhibited by non-secretor saliva belonging to the O and A_1 groups.

3) The hemagglutinins present in seeds of *Cytisus sessilifolius* are probably so-called anti-H agglutinins.

REFERENCES

1. Bernstein, F.: Klin. Wchnschr. 1924:*3:*1495.
2. Boorman, K., Dodd, B. & Gilbey, B. E.: Ann. Eugenics. 1948:*14:*201.
3. Eisler, M.: Z. Immunit. forsch. 1930:*67:*38.
4. Grubb, R.: Acta Path. et Microbiol. Scandinav. 1949:Suppl. 84.
5. Hirszfeld, L.: Erg. Hyg. usw. 1934:*15:*54.
6. Johnsson, B.: Acta Path. et Microbiol. Scandinav. 1941:Suppl. 54: 456.
7. Koulumies, R.: Ann. Med. Exp. Biol. Fenn. 1949:*27:*185.
8. Landsteiner, K. & Levine, P.: J. Immunol. 1926:*12:*441.
9. Landsteiner, K. & Witt, D. H.: J. Immunol. 1926:*11:*221.
10. Morgan, W. T. J. & Waddel, M. B. R.: Brit. J. Exper. Path. 1945:*26:* 387.
11. Morgan, W. T. J. & Watkins, W. M.: Brit. J. Exper. Path. 1948:*29:* 159.
12. Moureau, P.: Rev. d'hématol. 1946:*1:*266.
13. Putkonen, T.: Acta Soc. Med. Fenn. Duodecim 1930:ser.A:14:Fasc. 2.
14. Renkonen, K. O.: Ann. Med. Exper. Biol. Fenn. 1948:*26:*66.
15. Renkonen, K. O.: Ann. Med. Exp. Biol. Fenn. 1950. In press.
16. Schiff, F.: Klin. Wchnschr. 1927:*6:*303.
17. Thomsen, O., Friedenreich, V. & Worsaae, E.: Acta Path. et Microbiol. Scandinav. 1930:*7:*157.
18. Wiener, A.: Blood Groups and Transfusion. 3 Ed. Springfield 1946.
19. Wiener, A., Oremland, B. H., Hyman, M. A. & Samwick, A. S.: Am. J. Clin. Path. 1941:*11:*102.
20. Witebsky, E. & Klendshoj, N. C.: J. Exper. Med. 1941:*73:*655.

FROM THE STATE SERUM INSTITUTE, HELSINKI

TETANUS FOLLOWING VACCINATION AGAINST VARIOLA[1]

By

TAPIO SAVOLAINEN

(Received for publication March 15, 1950)

Vaccination against smallpox has become compulsory in many countries, among them Finland, where every child must be vaccinated during his second year.

Complications from vaccination are relatively scarce. Among them are enlisted: strong reactions to vaccination, encephalitis and, very seldom, tetanus. However, reports on at least 127 cases of post-vaccinal tetanus can be found in the Anglo-American literature alone (3 and 8). Several investigations on this complication have also been published in Europe (i.a. 1, 4 and 10).

Since, however, not a single case of this nature has been reported in Finland, and since the bacteriological verification is quoted in relatively few foreign publications (i.a. 1, 4 and 10), a report of our case seems justified.

A.T. a one-year 10 month old son of a glazer in Helsinki was inoculated against smallpox in his upper left arm on May 14, 1949, his general condition having been good before. A few days later a strong reaction appeared on the site of vaccination, accompanied by high temperature, which gradually decreased. Later pustules appeared also elsewhere on the skin, although the parents denied that the patient had scratched the site of vaccination, which first was covered by a piece of bandage fastened by

[1] In this connection I wish to thank the Chief Physician of the Hospital of Contagious Diseases in Helsinki, Professor V. Rantasalo, who has kindly given me access to the hospital record of the patient.

adhesive plaster and later by a proper bandage. The patient also had diarrhea and was taken to the Hospital of Contagious Diseases in Helsinki on May 30, 1949. The general condition of the patient was good, although he was pale and tired; his weight was 13 kilograms and length 83 centimeters. On the upper left arm was an area of 4 × 4 cm covered with purulent crust and surrounded by small pustules, either isolated or confluent. Purulent matter oozed forth from the whole area. On his right thumb was a pustule of 8 × 8 mm in addition to other pustules elsewhere: one on the back surface of the left leg, one on the lower left eyelid and one at the root of the nose. There were a few small reddish spots on the skin on the front head, the leg and the scalp. The patient had a temperature of 37.4° C. Gram staining of the smear taken from the skin yielded the following results: Gram negative rods, Gram positive staphylococci. On June 3, 1949 the temperature of the patient was normal but he suffered from trismus and later in the day had several attacks of convulsion, during which complete opisthotonos developed. On account of the clinical symptoms of tetanus the patient was given tetanus antitoxin and sedatives but the symptoms continued. Death occurred at 19.30 on June 3, 1949. Full and clear consciousness prevailed until the end and no rise in body temperature was observed.

In the lumbar puncture performed on June 3, 1949 the cerebrospinal fluid was clear, 0.3 cells per cu.mm, Pandy−, Nonne −, Bacteria −. During the whole period of hospitalization penicillin was administered.

The clinical diagnosis was: *Vaccinia. Tetanus.* Post-mortem diagnosis was: Reactio vaccinationis brachii sin. Vaccinia cutis capitis et palpebrae inf. oculi sin. et cutis parietis anterioris thoracis l. sin. Oedema cerebri (Encephalitis?). Hypostatis pulmonum. Fibrosis endo- et pericardii. Hyperaemia hepatis et renum. Hyperplasia lienis et lymphonodorum mesenterialium.

Bacteriological Investigation. — From the site of vaccination where the necrosis was observed to extend to a depth of 1½ cm a sample of tissue was taken for bacteriological investigation from the border between healthy and necrotic tissue. The remaining amount in the vial with vaccine used for the vaccination not only of this child but of 250 other children, but without any other cases of complications, was also taken for bacteriological investigation.

Under sterile conditions a piece of tissue the size of a pea was suspended into 2 cc of physiological saline solution. The coarse particles were filtered from the suspension and 0.1 cc of the obtained suspension was administered by intraperitoneal injection on to two white mice, in addition to which the other was given 0.2 cc or 200 units of tetanus antitoxin (Statens Seruminstitut, Copenhagen).

The test mouse died in less than 20 hours, the control animal was still spry and free from symptoms after 30 days.

Both aerobic and anaerobic cultures were performed of the pulverized tissue piece, the latter according to Zeissler's (13) method. In stainings of the abundant growth of bacteria in these cultures Gram-positive Staphylococci were observed, which hemolyzed on blood agar and were definitely of a *Staphylococcus aureus* type, as well as thin Gram-positive rod bacteria which rather slowly formed terminal round spores, fully corresponded morphologically to tetanus bacilli, were later found to be obligatory anaerobes and could easily be isolated. Animal tests with two pairs of mice using one day old liver broth culture of the pure strain were carried out in the following manner: 0.1 ccm of a dilution of the above-mentioned culture (one normal loopful of culture in 0.5 cc physiological saline solution) was administered to one pair, in addition to which one mouse in the pair received 0.2 cc of tetanus antitoxin. The test mouse died, having had convulsions, after 2 days 19 hours. The control mouse remained spry 30 days.

The second pair received 0.1 cc each of the same culture, but more diluted (one normal loopful in 2.0 cc of physiological saline solution), and the control mouse in addition 0.2 cc of tetanus antitoxin. The test mouse died after 3 days 19 hours, the control mouse remained spry 30 days.

The whole amount of the remainder of the vaccine used was investigated in several small portions, without any tetanus bacilli being discovered. On the other hand, however, growths occurred in every culture of Gram-positive Staphylococci of a *Staphylococcus albus* type which did not hemolyze on blood agar.

DISCUSSION

It has been possible to demonstrate by bacteriological investigation of a piece of tissue from the site of vaccination, where the tetanus infection obviously commenced, a strain of tetanus bacilli pathogenic to mice and *Staphylococcus aureus*, known to enhance the pathogenetic effect of tetanus bacilli (9). It is not possible, however, on the basis of the investigations performed to state the source of the infection: Was it the vaccine or was the infection secondary, or post-vaccinal?

The probability of presence of tetanus bacilli or their spores in the vaccine can indeed not be excluded.

In the first place, tetanus bacilli are common in the soil and in the intestines of domestic animals (12) and obviously (at least temporarily) on skins fouled by their excrements. Secondly, it is impossible to render human skin anywhere near free from bacteria by mechanical cleaning (11). Considering the method of preparing vaccine, the presence of tetanus bacilli in it is possible, wherefore it is nowadays endeavoured to eliminate this possibility by careful control investigations.

In the literature the author has found only one report of tetanus bacilli having been discovered in smallpox vaccine. Wilson reports (Lancet 1902:*2*:735) that he had discovered tetanus bacilli in large numbers in a vaccine virus made about the time of the epidemic of 1901; this strain had been pathogenic to small laboratory animals. On the other hand there are several negative investigations. Thus Anderson (2) investigated a quantity of vaccine which would have sufficed to vaccinate 2,000,000 people without discovering tetanus bacilli. Also Armstrong (3) refers to negative vaccine investigations.

Although post-vaccinal tetanus is a very rare complication a great number of investigations concerning it have been published. Not counting the relatively numerous investigations concerning individual cases, in which it has not been possible to demonstrate the infection mechanism (i.a. 4, 5, 6 and 10), the examination of certain more extensive series (3 and 8) indicates plainly a secondary, or post-vaccinal infection. Thus Armstrong (3) whose series comprised 98 cases and McFarland (8) who had 95 cases, found the average incubation time of tetanus following upon smallpox vaccination to be 18—24 days, but the death rate to be 75—80 per cent. In investigations of traumatic tetanus it has been possible to observe that the death rate decreased as the incubation time increased (7). As compared with traumatic tetanus the above-mentioned high death rate presupposes an incubation time half as long, i.e., the infection must have been post-vaccinal. In the case presented here the incubation time was 20 days, that is, the same as the above-mentioned average time, in view of which the infection obviously also in this case was post-vaccinal. The probability of secondary infection is further supported by the fact that no tetanus

bacilli were found in the remainder of the vaccine as well as the circumstance that 250 other children had been vaccinated with the same vaccine without a single case of tetanus.

It deserves to be noted that both Armstrong and McFarland were able to demonstrate that post-vaccinal tetanus occurred almost exclusively in patients whose inoculation wounds had been covered with bandages or plasters of various kinds, as in the case reviewed above, whereas tetanus had not occurred in openly treated wounds. The technical performance of the vaccination did not seem to have any effect on the incidence of tetanus.

Armstrong also proved his observations with animal tests by vaccinating a series of test animals and infecting the sites of vaccination with tetanus spores. After this half of the series was treated openly, the inoculation wounds of the other half were covered. It was observed that the latter animals contracted tetanus, whereas the openly treated animals did not.

The literature mentions certain figures on the danger of post-vaccinal tetanus. Thus, 585,000 persons were vaccinated in the U.S. Navy during the period 1904—1913 without a single case of tetanus. Lancet (1902:*1*:607) mentions the risk as 1: 5,000,000. Obviously this danger can be reduced by treating the wounds openly (3 and 8). The European fashion of vaccinating children as young is more advantageous than the American system of vaccinating at an age of 6—7 years (3), because with it the danger of secondary infection, greater for boys than girls, diminishes.

The author is informed of very few cases in our country of serious post-vaccinal complications, mainly encephalitis, many of which have been obvious cases. As no bacteriological investigations have been performed the possibility of some case of tetanus among them cannot be excluded.

SUMMARY

1) The writer presents a case of tetanus in a two year old boy following upon smallpox vaccination with an incubation time of 20 days, and which ended fatally in less than 24 hours after the first symptoms of the disease.

2) The origin of the disease was obviously the site of vaccination. From a piece of tissue excised from it was isolated a strain

of tetanus bacilli pathogenic to mice as well as a strain of *Staphylococcus aureus* known to enhance the pathogenetic effect of the tetanus bacillus.

3) From the remainder of the vaccine *Staphylococcus albus* was found but no tetanus bacilli.

4) A post-vaccinal secondary infection is probably in question here.

5) The adhesive plaster bandages and the other bandages used on the site of vaccination may have promoted the contraction of tetanus.

REFERENCES

1. Ambrosioni, P.: G. Batt. Imm. 1940:*24:*70.
2. Anderson, J. F.: Publ. Health Repts. (U.S.) 1915:*30:*2111.
3. Armstrong, C.: J.A.M.A. 1928:*90:*738.
4. Colo, F.: Med. Prat. 1932:*6:*418 quoted by Ambrosioni.
5. Findlay, W., and Findlay, J. W.: Lancet 1902:*1:*506.
6. Harte, R.: Ann. Surg. 1902:*35:*520.
7. Kurttio, E.: Über Tetanus und sein Vorkommen in Finnland. Helsinki. 1935.
8. McFarland, J.: Lancet 1902:*2:*730.
9. Mackie, T. J., McLaughlan, D. G. S., and Anderson, E. J. M.: Brit. Med. J. 1929:*2:*1167.
10. Muggia, —.: Il lattante 1930:*1:*617 quoted by Ambrosioni.
11. Saarenmaa, E.: Investigations of the Bacterial Flora, Disinfection and Disinfectants of the Skin. Helsinki. 1946.
12. Wilson, G. S., and Miles, A. A.: Topley and Wilson's Principles of Bacteriology and Immunity, London. 1947.
13. Zeissler, J.: Anaërobenzüchtung. Jena, Berlin und Wien 1930 (Handbuch der pathogenen Mikroorganismen) *10:*35.

FROM THE STATE SERUM INSTITUTE, HELSINKI

STERILITY OF CATGUT

By

TAPIO SAVOLAINEN

(Received for publication April 3, 1950)

The cases of postoperative tetanus in Finland after World War II in which catgut was the source of infection (19) necessitated a change from occasional investigations on sterility to systematic sterility control outside the factories of catgut manufactured in or imported to Finland. Such a control system has earlier been made compulsory in several countries, e. g., the United States, England and Germany (5). In these countries 1 ½ per cent of the length of the catgut is investigated, in Finland at least 1 per cent.

A feature common to the catgut packages of different factories seems to be the word »sterile» appearing in one way or another on the carton. The literature concerning investigations on sterility outside the factories does not seem to be very extensive. Thereare, however, a number of scientific investigations which have revealed a lack of sterility in the catgut, in most cases quite considerable. Thus, in an investigation on the catgut of eight English and American factories bacteria were found in 77 per cent and none of these products were sterile in all the tests (2). In another particularly extensive series of investigations, comprising 44,664 metres of catgut of German and other origin, it was found that 80.7 per cent of the large packages (coils or rolls of 50 metres) and 54.3 per cent of the small packages of the German catgut, and 57.2 per cent of the small packages of catgut of other origin were unsterile (15). According to a Danish investigation (4), in which the material

of 1,100 metres came from altogether nine factories, 62.3 per cent of the catgut was unsterile. In a German investigation, with a material of 1,400 metres, the unsterility percentage was as high as 80 (13).

According to comparative American investigations, the unsterility percentages of catgut manufactured in different countries were as follows: English 29.5, French 13.3, German 85.7, Japanese 100 and Spanish 63.1, or on the average, 46.5 (7, 8). In other words, according to these investigations 50 per cent of American and 62.5 per cent of the factories of other countries made unsterile catgut.

It was found in investigating small amounts of catgut kept in closed ampoules that the sterility was much better. According to certain American investigators (1, 17) only between 3 and 12.5 per cent of the total were unsterile.

A great number of sterilisation methods using many different kinds of chemicals have been developed to kill the anaerobes in the raw material of catgut, sheep gut, of which especially the spore-forming are extremely difficult to destroy. Although sterilisation methods have steadily improved, perfection has not been achieved. It has been demonstrated that spore-forming anaerobes can for years resist disinfectants regarded as effective (3). A great many methods of chemical sterilisation have furthermore been carefully examined and it has been possible to demonstrate that they are ineffective to such an extent (2, 6, 10 and 11) that strong demands have been raised for abandonment of all chemical sterilisation. Results achieved in Finland (19) confirm these results. In view of this the method of heat sterilisation has been or is being quite commonly taken into use, which by certain investigators (7, 11 and 18) is regarded the only method by which, with careful performance and control, sufficient sterility is achievable.

Abreast with the development in methods of sterilisation progress has been made in the investigation of sterility. The first good method was presented by Bulloch (2) in 1929 and a still better one in 1931 by Meleney and Chatfield (18). Variations of the latter are still in use in different parts of the world (1, 5, 9 and 19). Essential to all these methods is that chemicals inhibiting bacterial growth are removed from the catgut by neutralisation prior to culture. Clock ((9, 11) analysed carefully the catguts chemically before sterility investigations and developed on the

qasis of the analysis results the simple neutralisation liquids needed. Cultures are usually performed in test tubes in which it is easy to provide a high layer of culture medium to promote anaerobic growth. American investigators have in addition to this used a vaseline-paraffin seal on the surface of the culture medium to prevent access of oxygen. As regards the control time, some investigators consider a 10 day incubation time sufficient (14), others favoring a longer period, either 15 days (1, 7, 18) or 21 days, (4, 5, 19). The possibility of secondary infection has been excluded by such control investigations as required. (Thus it is reported that one investigation comprising 4,500 metres of catgut was carried out successfully without air infection (5).)

TECHNIQUE EMPLOYED

The catgut investigations were performed in a special room in which the bacterial content of the air was reduced by ultraviolet light in addition to regular cleaning. In the beginning, the ultraviolet light came from a tube when no work was performed in the room; later all day. The investigators washed their hands thoroughly before starting work and used masks. The general sterility instructions of our institute were observed in other respects. Thus test tubes, pipettes and dishes were sterilized in heat for 2 hours (at 180° C), likewise long forceps and scissors. The culture media were sterilized in an autoclave (120° C).

The catgut samples were taken at random from the firm or hospital stores in unopened original packages by our Institute. During the investigations the packages and catguts were touched only with the aforementioned instruments which were sterilized between use by burning in a Bunsen flame.

The catgut was cut into pieces of approximately 2 cm directly into large test tubes, about one lineal metre into each, into which 10 cc of neutralisation liquid (1 per cent sodium thiosulphate and 1 per cent sodium carbonate in water solution) was added. The tubes were then kept for 48 hours in a thermostat at 37° C, after which the neutralisation liquid was replaced by distilled water and the tubes were kept in a thermostat for 24 hours. The tubes were closed with cotton wadding.

The catgut samples were then placed in culture medium on the bottom of the tubes, about ⅓ metres into each tube, and were incubated in a thermostat at 37° C for 21 days. Readings were taken at 1, 3, 7, 14 and 21 days, giving special attention to anaerobes. Gram staining was performed on each tube showing growth and if no obvious growth of anaerobes was observed, fractional heating of the cultures to 100° C was performed and subcultures were made. Identification of strains, in so far as it was performed, was done according to Zeissler's (20) method.

No seal was used on the surface of the culture media, but immediately preceding culture the culture medium tubes were placed in a water bath at 100° C for at least 10 minutes and then cooled down to +45° C.

In the large test tubes the culture media were in layers of at least 10 cm. As media were used: liver broth, soft agar and soft glucose agar[1], with a pH of 7.4—7.6. The tubes were closed by cotton wadding and after culture by a waxpaper cap around the top of the tube attached by a rubber band.

Controls. — 1. During the whole that time catgut was handled the bacterial content of the air was controlled by uncovered glucose blood agar and blood agar dishes which were incubated from 24 to 48 hours in a thermostat at 37° C, in addition to which part of the dishes were incubated in vacuum (20). A corresponding number of covered control dishes were treated in the same manner. On the open dishes there were from 0 to 10 colonies of bacterial growth, usually from 0 to 3 colonies, predominantly different cocci, in some cases mold or yeast fungi or spore-forming aerobic bacteria. On the covered bowls growth occurred in 0—2 colonies, in most cases 0 colonies.

2. Control of neutralisation liquid: 1 cc of neutralisation liquid was transferred by pipette into at least one test tube of each culture medium per each daily catgut investigation series. These tubes were incubated together with the cultures. (cf. growth control).

---

[1] Liver broth: per tube, 13 cc of broth and 3—4 cubical pieces of boiled cattle liver with a side of approximately 6 mm. Soft agar: 0.3 per cent agar in broth. Soft glucose agar: 1 per cent glucose added. Broth: 0.5 per cent NaCl and 1 per cent pepton (Parke-Davis) in meat infusion. Meat infusion obtained by extracting boneless and sinewless ground beef in water; 0.5 kilograms of meat will yield 1 litre of meat water.

3. Control of distilled water: 1 cc of distilled water was transferred by pipette into at least one tube of each culture medium per each daily catgut investigation series. These tubes were incubated together with the cultures.

4. Growth control: Two normal loopfuls of dilutions of 1:1,000 and 1:1,000,000 of 24 hour old *Cl. bifermentans* liver broth culture were inoculated into sterile culture medium tubes.

5. Adding neutralisation liquid into the culture medium tubes this control was performed in the above described manner to prove that the neutralisation liquid had no bacteriostatic effect.

6. Control of culture medium: All culture media were incubated for 21 days parallelly with the catgut cultures. These tubes were handled in exactly the same manner as the catgut culture tubes, except that no catgut was placed into them. Each catgut culture tube had its corresponding culture medium control tube. Growth was very seldom observed in the tubes of this control and it was always growth of aerobic bacteria, usually cocci.

7. A number of culture medium tubes were incubated without any treatment in connection with each series to prove that the culture media were sterile.

This method of investigating sterility differs from earlier methods especially in that the sterility of the catgut can by this method be observed in each metre, even fractions thereof, by means of which a clear and visual picture of the bacterial content of the catgut is obtained.

Another difference is the wide range of the culture medium control. It must be admitted that one culture medium tube per each catgut culture tube gives a particularly clear picture of the extent to which air and other secondary infections may occur.

The possibility of air-borne infection was considerably reduced by the use of ultraviolet light.

As the sterility investigations concern only from 1 to 1 ½ per cent of the length of the catgut, certainly about sterility remains in every case relative and it is confirmed only when large materials are investigated. Earlier methods of sterility investigation dealt with whole catgut packages, by which means it was possible to establish whether a package was sterile or not, but not how much living bacteria each package contained. This circumstance is, however, of essential significance as the investigations reported in

the literature indicate that it seems to be very difficult to obtain absolutely sterile catgut, at least for the time being. It has also been demonstrated in the literature that living bacteria may be present in catgut on long distances from one another (12).

THE INVESTIGATED MATERIAL

A total of approximately 8,000 metres of catgut manufactured by 30 different factories, part of it sterilized chemically, part by heat, were investigated. Since the amounts of investigated catgut of most factories were so small that it was not possible to draw any definite conclusions from the results, only the results gained in investigations of catgut of eight factories will be discussed here. This material comprises 6,628 metres divided on 757 packages. Rolls and coils of 25—50 metres were counted as large packages and rolls and coils of 10 metres, of which there were only a few, other small packages containing 7.5 metres of catgut and ampoule packages containing 1.5—2.5 metres per ampoule, as small packages. The ampoules comprised the majority of small packages. With the exception of the ampoules all packages were original factory cardboard cartons. Most of them were manufactured recently or during the past few years, but some of them were over 10 years old. The method of sterilisation used by the factory was not reported in all cases.

RESULTS

In appreciating results, bacterial growth was counted per catgut metre. Attention was given to both anaerobic and aerobic bacteria in counting the percentage of unsterility of the catguts. This percentage was counted on packages and also by taking one metre as unit. In the latter case, for example if there has been growth of bacteria in five of 200 investigated metres, the percentage of unsterility is 2.5, and so on.

Results are given in the below table (Table 1).

A study of table 1 reveals that 3.5 to 100 per cent of the catgut packages of different factories were unsterile, which figures correspond to earlier results.

The use of both kinds of chemically-sterilized catgut shown in the table, Swiss and Finnish, must absolutely be abandoned

TABLE 1

RESULTS OF BACTERIOLOGICAL INVESTIGATION OF CATGUT

Factory	Method of Sterilisation	Investigated						Results						
			Packages					Packages			Bacterial Growth			
			Large		Small									
		Metres	Whole	Partly	Whole	Partly	Total	Sterile	Unsterile	Percentage of Unsterile	Anaerobes in Metres	Aerobes in Metres	Total in Metres	Percentage of Unsterile Metres
English ..	Heat	1,970	31	18	96	—	145	108	37	25.5	42	62	104	5.28
Danish ..	Heat	1,070	15	26	—	—	41	29	12	29.3	17	9	25[1]	2.34
Dutch....	Heat	260	5	4	—	—	9	2	7	77.8	23	22	43[1]	16.54
Australian	Heat	480	—	—	315	—	315	304	11	3.5	7	4	11	2.29
German I	?	305	1	33	9	54	97	77	20	20.6	16	42	55[1]	18.03
German II	?	185	—	26	14	17	57	53	4	7.0	7	4	11	5.95
Finnish ..	Chem.	2,225	43	43	—	—	86	32	54	62.8	94	182	258[1]	11.59
Swiss	Chem.	133	2	—	2	3	7	—	7	100.0	110	78	126[1]	94.70
		6,628	97	150	436	74	757	605	152		316	403	633	

[1] As both anaerobic and aerobic bacteria have grown from the same catgut metre the number of unsterile metres is not the same as the sum of metres with anaerobic and aerobic growth, but smaller.

(6, 19). Of the former there was not a single sterile package and the percentage of unsterile metres was as high as 94.70. Of the latter, 62.8 per cent of packages were unsterile and the percentage of unsterile metres was 11.59.

Of the catguts sterilized by heat, that produced in Holland could not be used because it had an unsterility percentage of 77.8 in respect to packages and of 16.54 in respect to metres. The same was the case with the German I catgut. The percentage of unsterility in respect to packages was, to be sure, only 20.6, but in respect to metres it was 18.03. The circumstance that these figures are almost equal is due to the fact that most of the investigated packages were small and only a small part of the large packages were investigated.

In respect to sterility, Danish, Australian, German II and English catguts were superior to the others. The degree of sterility of the catguts of these factories seems, however, to differ considerably depending on whether whole packages or catgut by metres are

investigated. With the latter method the order of sterility is as follows: Australian 2.29, Danish 2.34, English 5.28 and German II 5.95 per cent unsterile.

If sterility is examined by whole packages, the order is as follows: Australian 3.5, German II 7.0, English 25.5 and Danish 29.3 per cent unsterile.

The difference which arises when the unsterility is estimated by packages or metres depends on the size of the packages. With small packages (1 ½—2 ½ metres) the difference is slight; the Australian catgut, for example, had an unsterility percentage of 3.5 in respect to packages and of 2.29 in respect to metres, the German II catgut correspondingly had the percentages 7.0 and 5.95, respectively. The Danish catgut, on the other hand, of which there were large packages only, had an unsterility percentage of 29.3 in respect to packages and of 2.34 in respect to metres, English catgut correspondingly had the percentages 25.5 and 5.28, respectively. If only the large packages of the English factory (containing a total of 1,800 metres of catgut) are considered, the unsterility percentage of packages rises to 58.8.

Investigation on the sterility of catgut per metre gives a clear picture of its unsterility irrespective of the size of the catgut packages. It also confirms the opinion, earlier presented in the literature (12), that living bacteria may be present in catgut at long distances from one another. Thus, during investigation of one of the above-mentioned catguts, anaerobic bacteria were found in 13 metres of one 50 metre roll after several hundred sterile catgut metres. It is in particular such observations which speak in favour of continuous sterility control outside the factory to discover weak spots in otherwise good catgut due to possible technical errors (as probably occurred in this case).

As regards the presence of anaerobic and aerobic bacteria in the cultures it seems as if especially the number of aerobic bacteria diminishes as the sterility of catgut improves. Thus, definitely more anaerobic than aerobic bacteria grew in the best qualities, the Australian, Danish and German II catguts (exception: English catgut), whereas in the poorer qualities, Finnish and German I, there was more growth of aerobic bacteria than of anaerobic (exception: the inferior Swiss catgut and the Dutch catgut which had slightly more growth of anaerobic bacteria).

If we take separately the catgut cultures with growth of anaerobes and those with growth of aerobes and examine the macroscopically visible growth of both, the time required and its distribution over 21 days in periods of one week, we receive the following table (Table 2):

TABLE 2

TIME NEEDED FOR MACROSCOPICALLY VISIBLE GROWTH OF ANAEROBES AND AEROBES AND ITS DISTRIBUTION IN PERIODS OF ONE WEEK

Time in Weeks	Growth of Anaerobes			Total	Growth of Aerobes			Total
	1	2	3		1	2	3	
No. of cultures with growth	301	51	52	404	347	61	46	454

A study of table 2 reveals that ¾ of growth in the cultures took place during the first week, irrespective of whether anaerobes or aerobes were in question. There were 404 cultures with anaerobic growth and 454 cultures with aerobic growth, or a total of 858 cultures. During the third week growth took place in 98 cultures, which number is about 13 per cent of the number of growths during the first and second weeks (760): i.e. with a three-week control time 13 per cent more positive results are obtained than with a two-week control time. The number of positive cultures decreases sharply after the first week, which trend is particularly clear in respect to aerobes. Subcultures of growths during the first week succeeded well, whereas subcultures of anaerobic bacteria which grew during the third week failed or produced very little growth. Judged on this basis a 21 day control time seems sufficient.

The speed of growth of aerobes was definitely greater than that of the anaerobes. The aerobes had an obvious peak at about three days, whereas, discounting the Swiss catgut which had a particularly rapid growth, the anaerobes had no definite growth peak, but on the contrary a slight decline after the first day.

It may be added that there were knots in 35 of the anaerobic cultures but in only 15 of the aerobic cultures. It is known that sterilization of knots in catgut is very difficult (5).

A study of the distribution between different culture media of the anaerobic and aerobic bacteria which have grown from catgut yields the following table (Table 3):

TABLE 3

DISTRIBUTION OF ANAEROBES AND AEROBES BETWEEN DIFFERENT CULTURE MEDIA IN CATGUT CULTURES

	No. of Anaerobes Grown in				No. of Aerobes Grown in			
	Soft Glucose Agar	Soft Agar	Liver Broth	Total	Soft Glucose Agar	Soft Agar	Liver Broth	Total
No. of cultures with growth	250	124	30	404	139	209	106	454

Soft glucose agar is a good culture medium for anaerobes, considerably better than mere soft agar. Aerobes seem to grow well in both media; however, the growth is definitely better in soft agar. In that they are semi-solid, both culture media have the advantage that it is easy to observe possible aerobic bacteria infection on their surface. In liver broth, aerobes seem to have succeeded better. This may to some extent be imaginary, because it is no easy to task to separate anaerobes from spore-bearing aerobic bacteria. But in practice it is not the decisive factor whether anaerobes or aerobes have grown, but whether the catgut is sterile or not.

As regards the anaerobic bacteria found from catgut, there was not in every case an attempt to definitely identify them; a great number of sample tests, were, however, performed. A microscopic examination of Gram-stainings made from positive cultures gives a rough picture of the matter. Liver broth has in this respect been very valuable, also in primary cultures but especially in subcultures. In liver broth anaerobes grow quickly and soon from spores, by the size and location of which it is possible to roughly orientate oneself. The presence of, among others, the following bacteria, was established in detailed identification: *Cl. welchii*, *Cl. novyi*, *Cl. tetani* (the writer will discuss its presence in his next publication), *Cl. bifermentans*, *Cl. multifermentans*, *Cl. sphenoides* and *Cl. tertium*. *Cl. welchii*, *Cl. novyi* and *Cl. septicum* have earlier been isolated from raw catgut, but not tetanus bacilli, although their presence has not been denied (17). *Cl. bifermentans*, *Cl. fallax*, *Cl. histolyticum*, *Cl. welchii*, *Cl. sporogenes* and *Cl. oedematoides seu sordellii* have earlier been isolated from commercial catgut (11, 16).

The aerobic bacteria in the catgut cultures were not identified finally. It can, however, be mentioned that Gram-positive rods,

both spore-bearing and without spores, aerobes of diphtheroidtype aerob and aerobes of the actinomyces group grew in the cultures. Also yeast fungi and not seldom *B. mesentericus ruber*-type aerobes were present.

SUMMARY

1. The method used to control the sterility of catgut was essentially the same as those reported previously, but differed from earlier methods in that the range of the culture medium control had been extended and that the sterility of the catgut could be observed metre by metre, so that it was possible to obtain a clear and visual picture of the distribution of unsterility.

2. The investigated material comprised 6,628 metres of catgut divided on 757 packages. The catguts were manufactured by eight different factories of seven different countries. Part of the catguts were sterilized chemically, part by heat, whereas in a part of the material the method of sterilisation had not been reported.

3. The percentage of unsterility per catgut package varied between 3.5 and 100, or about the same limits as reported earlier.

4. The percentage of unsterility of investigated metres of catgut of different factories varied between 2.29 and 94.70. The per metre investigation of catgut gave a clear picture of the unsterility of the catgut irrespective of the size of catgut packages.

5. The sterility of chemically sterilized catgut proved to be poor. On the basis of the results of the investigations Clock's opinion that the use of chemically sterilized catgut should be completely abandoned must be supported.

6. Catgut sterilized by heat proved clearly better than chemically sterilized catgut. In the case of some factories it was to such extent unsterile that it was not dared to use it. As a rule the best catguts sterilized by heat were rather sterile showing only temporary deviations.

7. As the sterility of catgut improved it seemed as if especially unsterility due to presence of aerobic bacteria diminished.

8. 21 days was used as incubation time in the investigations and it could be proved that it yielded 13 per cent more positive results than a 14 day incubation time. Seventy-five per cent of the growth in positive cultures occurred during the first week. A 21-day control time was considered sufficient.

9. Soft glucose agar (0.3 per cent agar and 1 per cent glucose) proved to be rather a good culture medium for anaerobes. Liver broth was particularly valuable for definite identification of strains.

10. The following anaerobic bacteria, among others, were found from the catguts: *Cl. welchii, Cl. novyi, Cl. histolyticum, Cl. tetani, Cl. bifermentans, Cl. multifermentans, Cl. sphenoides* and *Cl. tertium*, as well as a number of undetermined aerobic bacteria, chiefly Gram-positive rods, spore-bearing and without spores, aerobes of a diphtheroid-type aerobes, aerobes belonging to the actinomyces group, as well as yeast fungi and aerobes of a *B. mesentericus ruber*-type.,

11. It is obvious, on the basis of the investigations performed, that continous control of sterility outside the factories is necessary, whatever method of sterilisation is used.

REFERENCES

1. Brewer, J. H.: J.A.M.A. 1937:*108*$_1$:722.
2. Bulloch, W., Lampitt, L. H. and Bushill, J. H.: The Preparation of Catgut for Surgical Use. Brit. Med. Res. Counc. Spec. Rep. Ser. 138. 1929. Quoted by Christensen.
3. Bulloch, W.: Zbl. Bakt. usw. 1 Abt. Orig. 1928:*106*:21.
4. Christensen, H. M., und Marcussen, P. V.: Acta Path. et Microbiol. Scand. (Danish) 1943:*20*:450.
5. Christensen, H. M.: Catgut Sterilization. The Basic Principles of the Spore-Germinating Method. Academic Dissertation, Copenhagen 1944.
6. Clock, R. O.: Surg., Gyn., & Obst. 1933:*56*$_1$:149.
7. Clock, R. O.: Surg., Gyn., & Obst. 1934:*59*$_2$:899.
8. Clock, R. O.: Surg., Gyn., & Obst. 1935:*60*$_1$:202.
9. Clock, R. O.: Surg., Gyn., & Obst. 1935:*61*$_2$:789.
10. Clock, R. O.: Surg., Gyn., & Obst. 1937:*64*$_2$:1027.
11. Clock, R. O.: Surg., Gyn., & Obst. 1938:*66*$_2$:878.
12. Mackie, T. J.: An Inquiry into Post-operative Tetanus. A Report to the Scottish Board of Health. Edinburgh. Quoted by Christensen, and Brit. Med. J. 1928:*1*:987.
13. Knorr, M.: Münch. med. Wschr. 1930:*1*:581.
14. Knorr, M.: Arch. Hyg. 1931:*105*:355.
15. Konrich, F., und Zeissler, J.: Arch. klin. Chir. 1933:*177*:742.
16. Meleney, F. L., Humphreys, F. B., and Carp, L.: Surg., Gyn., & Obst. 1927:*45*$_2$:775.
17. Meleney, F. L., and Chatfield, M.: Surg., Gyn., & Obst. 1930:*50*$_1$:271.
18. Meleney, F. L., and Chatfield, M.: Surg., Gyn., & Obst. 1931:*52*$_1$:430.
19. Savolainen, T.: Ann. Med. Exper. Fenn. 1950:*28*:55.
20. Zeissler, J.: Anaërobenzüchtung. Handbuch der pathogenen Mikroorganismen. Jena, Berlin und Wien, 3 Aufl. 1930:*10*:35.